# Molecular and Genomic Study of Genes Involved in Species Adaptation

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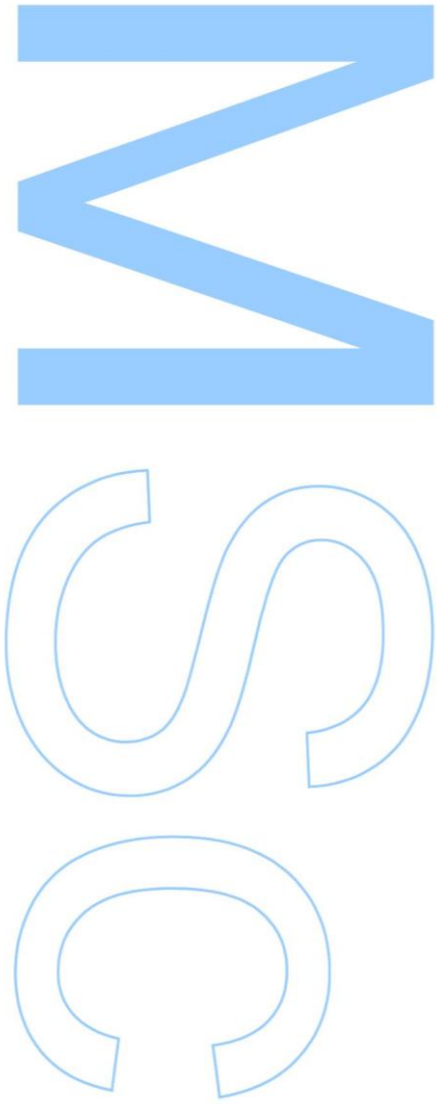

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# Sumário

Os mecanismos de adaptação das espécies ao meio ambiente estão intimamente relacionadas com a eficiência de genes codificantes de proteínas, o que resulta de modificações no número de cópias, estrutura e função desses mesmos genes. Estas alterações são comuns em genes codificantes de proteínas que influenciam o "*genetic fitness*", tal como os genes envolvidos na perceção sensorial ou processos de destoxificação.

Neste estudo foram analisados genes codificantes de proteínas que influenciam a adaptação dos vertebrados, incluindo os recetores envolvidos na deteção de feromonas, tais como o reportório de recetores vomeronasais em anfíbios, répteis e mamíferos, e os recetores olfativos relacionados com a classe A e C em peixes, os quais têm funções importantes nas relações intraespecíficas como acasalamento e cuidado parental. Também foram analisados genes que codificam proteínas de sistemas enzimáticos de destoxificação em espécies de aves (família das CYP2 e família das cGST) que têm papéis na defesa contra compostos endógenos e exógenos. Em ambos os casos, avaliamos as relações entre características genómicas observadas e a possível relevância para a adaptação das espécies.

Detetamos a presença de múltiplas cópias de genes sugestivas de adaptação destas famílias de genes para reconhecer uma grane panóplia de moléculas. Concluímos que os sistemas genéticos analisados são bons exemplos da plasticidade dos genes o que permite que as espécies se adaptem com sucesso a novas condições ambientais.

## Palavras-chave

Adaptação ambiental – Perceção Sensorial – Destoxificação

# Abstract

Mechanisms of species adaptation to the environment are closely related with the protein-coding genes performance that is a result of the modifications in copy number, structure and function of genes. These alterations are common in protein-coding genes influencing the genetic fitness, such as in genes involved in sensorial perception or in detoxification processes.

In this study we analyzed protein-coding genes influencing vertebrates adaptation, including the receptors involved in the detection of pheromones, such as the repertoire of vomeronasal receptors in amphibians, reptiles and mammals and olfactory receptors related to class A and C in fishes, which have important functions in intra-species relationships like mating and parental care. We also analyzed genes that codify proteins of enzymatic detoxification systems in avian species (CYP2 family and cGST family) playing key roles in the defense against both endogenous and exogenous compounds. In both cases, we assessed the relationships between observed genomic features and the possible relevance for the species adaptation.

We detected the presence of multiple gene copies suggestive of the adaptation of these gene families to recognize a high panoply of molecules. We conclude that the analyzed genetic systems are good examples of the genes plasticity that allowed species to successfully adapt to new environmental conditions.

## Key words

Environmental adaptation – Sensory perception – Detoxification

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# List of abbreviations

**19-(s) HETE** – 19-S hydroxyeicosatetraenoic acid
**cGST** – cytosolic GST
**CNG channel** - Cyclic nucleotide-gated ion channel
**CRs** - Chemosensory receptors
**CYP450** – Cytochrome P-450
**CYP2** – CYP450 family 2
**EET** – Epoxycosatrienoic acid
**GDP** – Guanine diphosphate molecule
**GPCRs** - G protein-coupled receptors
**GSH** – Glutathione
**GST** – Glutathione s-transferase
**GTP** – Guanine triphosphate molecule
**MOE** – Main olfactory epithelium
**MORs** - Main olfactory receptors
**mRNA** – messenger ribonucleic acid
**MT** – Metallothionein
**NADPH** – Nicotinamide adenine dinucleotide phosphate
**OlfC** - Olfactory receptor related to class C GPCR
**Ora** - Olfactory receptors related to class A GPCRs
**PAH** – Polyaromatic hydrocarbons
**PPK** channel – Pickpocket channel
**ROS** – Reactive oxygen species
**SOD** – Super oxide dismutase
**T1R** - Taste receptor type 1
**T2R** – Taste receptor type 2
**Taars** - Trace amine-associated receptors
**TRPC2** - Transient receptor potential channel 2
**UDP** - Uridine diphosphate glucose
**UGT** – UDP-glucoronyl transferase
**V1R** - Vomeronasal receptor type 1
**V2R** – Vomeronasal receptor type 2
**VNO** – Vomeronasal organ
**γ-GTP** – γ-glutamyltranspeptidase

# General introduction

The success of organismal lineages requires the viability of all stages of development, such as initial development and growth, in order to achieve sexual maturity and transmit their genetic information to the offspring. However this viability is closely connected with adaptability to the environment not only to promote profitable reproductive strategies but also to develop mechanisms that keep the organisms healthy. Adaptation processes, contributing to the genetic fitness, are crucial for species survival and are responsible for dynamic plasticity of protein-coding genes that result in alterations in their copy number, structure and function. Usually, constitutive protein-coding genes are more conserved and are under strong-to-moderate purifying selection, which favors the purging of occasional mutated alleles to retain gene funtionality. Nevertheless, protein-coding genes involved in genetic fitness processes are usually influenced by positive selection that favors the retention of mutations to improve protein function. These alterations contribute to the dynamic evolution of protein-coding genes involved in adaptive processes, such as sensory perception and detoxification.

In this work we studied the genomics of the sensory perception of pheromones, which have a crucial role in intra-specific relationships like mating and parental behaviour. As the correct pheromonal communication is an important step in species reproduction, the involved receptors have to be conserved to allow species-specific communication but also have to be plastic to recognize high panoply of molecules and be prepared to recognize putative changes in pheromones. Thus, we assessed the evolutionary genomics of receptors involved in pheromonal detection across vertebrates, obtaining insight how environmental pressures may have influenced their evolution.

We also studied protein-coding genes involved in detoxification processes that are crucial to metabolize endogenous and exogenous toxic compounds. CYP450 and GST enzymatic families, involved in detoxification Phase I and Phase II, respectively, are connected since GST enzymes metabolize products resulting from some CYP450 elements activation. The main goal was to study and understand how genomic changes in these two correlated detoxification enzymatic systems are related with their biologic function and species fitness.

# Chapter A – Sensory perception

## Introduction

Recognition of odors and chemical substances is extremely important for the survival of animals because it allows choosing appropriate food (avoiding consumption of toxic products), escaping predators, kin recognition and finding breeding partners for reproduction (Hino, Miles et al. 2009, Dong, Jin et al. 2012, Johnstone, Luibieniecki et al. 2012).

In vertebrates, the odors are detected by chemosensory receptors (CR), which belong to the family of G protein-coupled receptors (GPCR) with characteristic seven transmembranar domains (highly hydrophobic regions that spans the membrane) (Dong, Jin et al. 2012, Johnstone, Luibieniecki et al. 2012), which make them structurally similar (Figure 1). The N-terminus region is located on the extracellular side of the membrane, while the C-terminus is located in the cytoplasmic side and interacts with other signaling molecules. Three extracellular loops alternate with three intracellular loops linking the seven transmembranar regions.

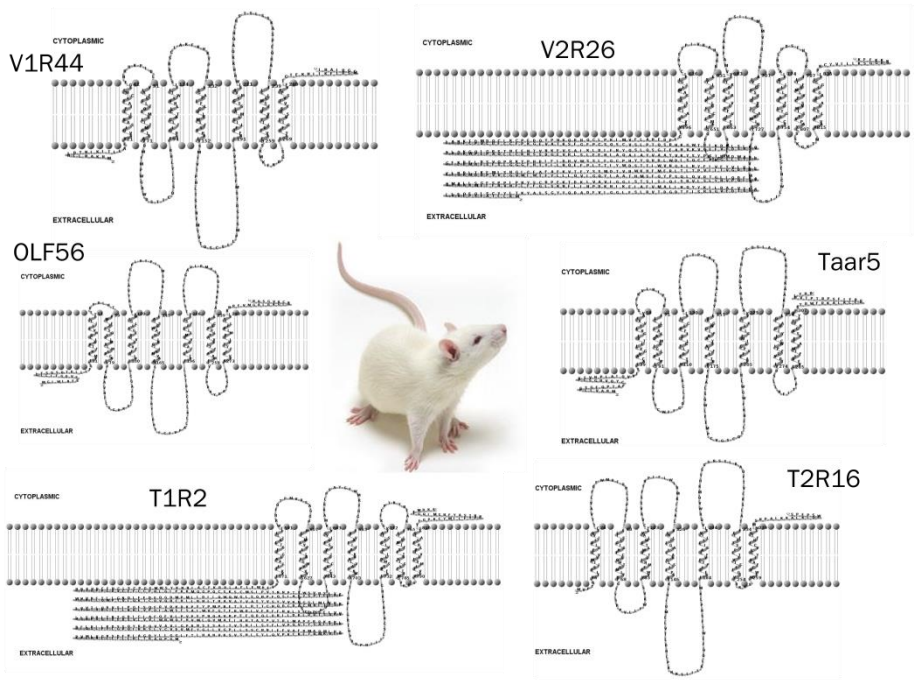



Figure 1 – Structure of representative chemosensory receptors present in mouse. V1R44, V2R26, OLF56, Taar5, T1R2 and T2R16 sequences and available information were retrieved from Uniprot database (IDs Q9EQ47, Q6TAC4, G8VGD6, Q5QD14, Q925I4 and P59529, respectively). The 2D graphical models of each receptor were constructed with TMRPres2D tool (Syropoulos, Liakopoulos et al. 2004).

Chemosensory receptors (CRs) are diversely encoded in animal genomes and can be subdivided in six different multi-gene families: main olfactory receptors (MORs), vomeronasal receptor type 1 and type 2 (V1R and V2R), trace amine-associated receptors (Taars) and taste receptor type 1 and type 2 (T1R and T2R) (Dong, Jin et al. 2012) (Figure 1). T1Rs and T2Rs genes codify gustation receptors with expression in taste buds of the tongue (Bachmanov and Beauchamp 2007). MORs, V1Rs, V2Rs and TAARs genes encode odorants receptors with MORs and TAARs being mostly expressed in main olfactory epithelium (MOE) whereas V1Rs and V2Rs are mainly expressed in the vomeronasal organ (VNO) (Dong, Jin et al. 2012).

Despite the diversity of receptors involved in odors detection, both are connected with olfactory neurons that have long thin cilia offering a large surface area for the interaction with odorant signals. In general, olfactory stimulus, which is diffused in the air, contacts with the mucous layer of the nasal cavity and binds any CR. CR interacts with intracellular hetrotrimeric G-protein (composed by α, β and γ subunits). In resting state, α-subunit of G protein bound guanine diphosphate (GDP) molecule but the interaction between odorant molecule and CR leads to conformational alterations in receptor that facilitates the exchange of GDP by guanine triphosphate (GTP) molecule and consequently the activation of G-protein.

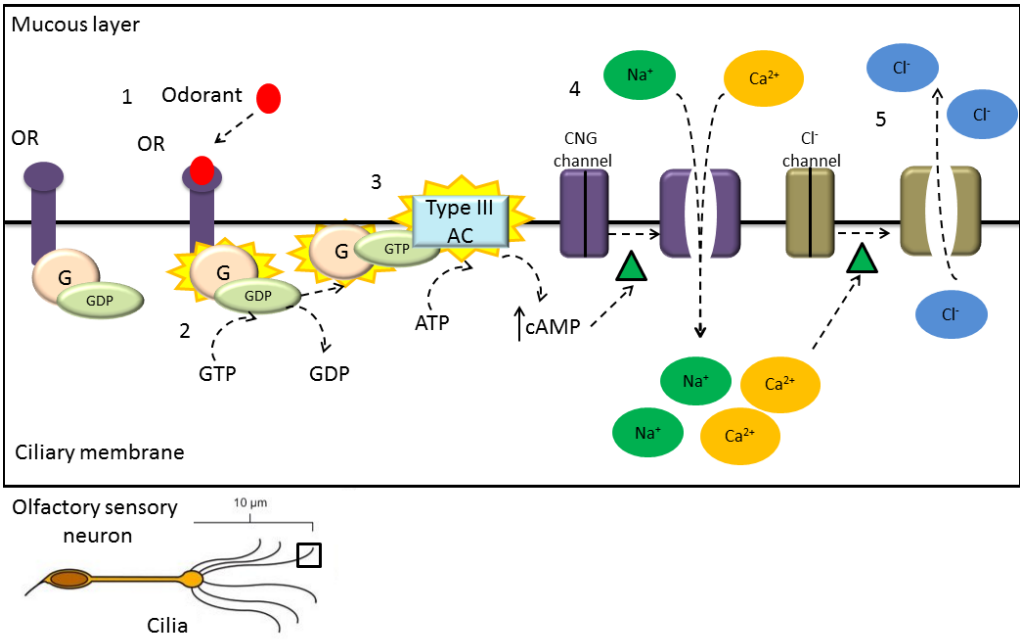



Figure 2 – Molecular events in odorants detection. When odorants arrive to mucous layer, they can interact with olfactory receptor (OR) (1) which leads to activation of heterotrimeric G-protein by replacement of GDP by GTP (2). Active G-protein activates type III adenylate cyclase that converts ATP in cAMP (3). cAMP induce the opening of the CNG channels, allowing the entering of $Na^+$ and $Ca2^+$ that will depolarize the neuron (4). In addition, $Ca2^+$ also induces the $Cl^-$ channel opening to release negative charges and potentiate the depolarization process (5).

This replacement facilitates the dissociation of active G-protein from the receptor, which allows the type III adenylate cyclase activation. Active adenylate cyclase will convert ATP into cAMP to increase its levels.

This phenomenon opens olfactory specific cyclic nucleotide-gated channels (CNG channels) at ciliary membrane that allow $Ca^{2+}$ and $Na^{+}$ influx. This small increase of positive charges leads to a small depolarization, known as receptor potential. However, intracellular $Ca^{2+}$ also induces calcium-activated chloride channels, which allow $Cl^{-}$ efflux and potentiate the depolarization process. If a huge number of molecules and receptors interact, the receptor potential will be strong enough to create action potential in olfactory neuron (Figure 2). Then, the brain will integrate all of these inputs to discriminate the odor and register the smell (Nelson and Cox 2008, Hino, Miles et al. 2009).

One of the most well studied chemical odorant substances are pheromones. Pheromones were identified in 1950s as “substances secreted by an individual to the outside, being perceived by another individual of the same species and causing the release of a specific reaction” (Karlson and Lusher 1959), changing behaviour and physiology (Shi and Zhang 2007) in ways like sexual recognition, mating (Chamero Leinders-Zufall et al. 2012) or neuroendocrine responses (Dong, Jin et al 2012). Despite similar main features, insects and worms usually have long chain hydrocarbons and ascarosides molecules, respectively, as pheromones, while in vertebrates the main intervenient are small proteins or peptides (Gomez-Diaz and Benton 2013). Pheromones are also specific chemical structures since the pheromones stereoisomers are able to block the pheromone activity (Mori 1997).

In addition to pheromones, species also use kairomones that are chemical cues, similar to pheromones, with heterospecific detection (Koh and Carlson 2011). Kairomones communication is widespread among vertebrates and invertebrates with important role in host and ectoparasites relationship, such as attraction of tick *Amblyomma americanum* by acid uric excreted for reptiles and birds (Rajchard 2013). Kairomones also affect the predator-prey relationship, giving disadvantages for the signaler and advantages for the receiver (Koh and Carlson 2011), as for example in the Eurasian otter. The main diet of Eurasian otter is composed by salmons but chemical cues released by otters are detected and recognized by young salmons, which allowed them to recognize the predator (Rajchard 2013). Among invertebrates, kairomones could be used in control of pests. One practical example is the construction of traps with the lizard *Varanus niloticus* kairomones that attract the vector of sleeping sickness fly *Glosina fuscipes fuscipes* (Aksoy, Omolo et al. 2009).

In 1990s it was firstly identified the vomeronasal receptors in rodents, as also their main role in pheromones detection (Dulac and Axel 1995, Herada and Dulac 1997, Matsunami and Buck 1997, Ryba and Tirindelli 1997). Currently it is consensual that, in the majority of vertebrates, pheromones and some kairomones (Papes, Logan et al. 2010, Koh and Cralson 2011) are detected by vomeronasal receptors which do not exclude the possibility of vomeronasal system detect non-pheromonal stimulus and/or some pheromones elicit responses in other systems (Baxi, Dorries at al. 2006). The existence of the vertebrate vomeronasal system, whose main function is pheromone detection, contrast with the scenario in insects where volatile pheromones are usually detected by odorant receptors (Benton 2008), while less-volatile pheromones might be detected by gustatory receptors and/or pickpocket (PPK) ion channels (Gomez-Diaz and Benton 2013).

The main objective of this chapter is the genomic study of the pheromonal communication and the pheromonal detection systems present in vertebrates. We assessed the genomic distribution of vomeronasal receptors across tetrapods to infer if their evolution is influenced by environmental/social pressures, such as water to land species transition, domestication processes or evolution of other sensory systems. We also studied the relationship between presence and/or size of vomeronasal apparatus and the expansion of vomeronasal receptors repertoire. Moreover, we further evaluate the possibility of detecting vomeronasal receptors in genomes of species that were previously reported to lack this sensory system. In basal vertebrates, such as fishes, as the vomeronasal system is absent we focus our genomic searches in alternative molecular systems involved in pheromonal detection, such as Ora and OlfC gene families.

## Material and methods

### Chemosensory genes structure

Representative sequences of mouse chemosensory receptor genes were retrieved from Uniprot database (V1R44 (Q9ED47), V2R26 (Q6TAC4), T1R2 (Q925I4), T2R16 (P59529), Taar6 (Q96RI8), OLF56 (Q8VGD6)). Available information about protein regions, such as transmembranar domains or signal peptide regions, was considered. The 2D graphical models of each receptor were constructed with TMRPres2D tool (Spyropoulos, Liakopoulos et al. 2004). PROSITE database was used to detect conserved motifs in all the sequences (Sigrist, de Castro et al. 2013). In

addition, one representative sequence of the mouse V1R and V2R family was retrieved from the Ensemble database (Vmn1r45 ID: ENSMUSG00000044248| ENSMUSTG00000044248 and Vmn2r26 ID: ENSMUSG00000096630| ENSMUSTG00000032238). Available information regarding the location of genes, genes structure and its transcripts was collected and for each sequence the DAS-TM filter server (Cserzo, Eisenhaber et al. 2002, Cserzo, Eisenhaber et al. 2004) was used to predict transmembranar domains. Transmembranar domains were annotated using Prosite MyDomains image creator (http://prosite.expasy.org/cgi-bin/prosite/mydomains/).

TRPC2 synteny

Genomicus genome browser was used to compile Ensembl information about the TRPC2 location and also their flanking genes. The synteny of the TRPC2 gene was analyzed in mammals, birds, reptiles, amphibians and fishes.

Vomeronasal receptors in vertebrate genomes

Ensembl and NCBI database searches for the V1R and V2R genes in the genomes of birds, reptiles, cetaceans and bats were firstly conducted using key-words (e.g. vomeronasal, VR, among others). Available sequences annotated as vomeronasal-like were detected and retrieved for reptile, cetacean and bat species. Whole genome sequences (WGS) searches were conducted inside birds WGS sequences using reptilian V1R-like sequences (XM_005291513.1 and XM_006020910.1) whereas WGS searches for mammalian species were conducted using Ensembl repertoire of cat V1R sequences (IDs in Supplementary file 1). The identity of all putative sequences were confirmed by Blast searches and discarded if the strong hit was not a vomeronasal receptor. Transmembranar regions were predicted by DAS-TM filter server (Cserzo, Eisenhaber et al. 2002, Cserzo, Eisenhaber et al. 2004) and conserved domains were searched inside PROSITE database (Sigrist, de Castro et al. 2013). The 2D graphical models of each receptor were constructed with TMRPres2D tool (Spyropoulos, Liakopoulos et al. 2004).

Identification of Ora genes in fishes and phylogenetic analysis

We performed searches of the Ora genes in 11 fish genomes available in Ensembl database (*Astyanax mexicanus, Danio rerio, Gadus morhua, Latimeira Chalumnae, Lepisosteus oculatus, Oreochromis niloticus, Oryzias latipes, Petromyzon marinus, Takifugu rubripes, Tetraodon nigroviridis* and *Xiphophorus maculatus*) and two further fish species from the NCBI database (*Danio rerio* and *Haplochromis chilotes*). The IDs of the retrieved sequences are in Supplementary file 2. Nucleotide sequences of Ora genes were aligned by codons using the program ClustalW2 inside SeaView5 package (Gouy, Guindon et al. 2012). Using the best-fitting model, GTR+I+G model (determined by jModeltest2 program (Guindon 2011, Darriba D., Taboada G.L. et al 2012), a Bayesian phylogenetic tree was constructed with MrBayes software version 3.2.2 (Ronquist and Huelsenbeck 2003). The reliability of our tree was assessed by bootstrap support until achieving a value of deviation of the split frequencies lower than 0.005 (analysis with total number of 385 000 generations). The first 25 percent of the results were discarded for the calculation of the posterior Bayesian probability.

Ora genes synteny

Genomicus genome browser was used to compile the Ensembl information on the fish Ora gene location and their flanking genes for *Astyanax mexicanus, Danio rerio, Gadus morhua, Latimeira Chalumnae, Lepisosteus oculatus, Oreochromis niloticus, Oryzias latipes, Petromyzon marinus, Takifugu rubripes, Tetraodon nigroviridis* and *Xiphophorus maculatus*.

OlfC genes cluster organization and OlfC phylogeny

Based on the available literature on the cluster organization of OlfC genes in Lake Victoria cichlids, Atlantic salmon and Zebrafish (Johnstone, Ciborowski et al. 2009, Nikaido,Suzuki et al. 2013), we have extended our study to medaka, tetraodon, platyfish, stickleback, cavefish and spotted gar, searching the Ensembl database and using the Genomicus browser server. Phylogenetic analyses were conducted with complete annotated OlfC sequences retrieved from the fish genomes available in the Ensembl database (IDs in Supplementary file 3). In our dataset we included the complete sequences of the annotated *Danio rerio* OlfC dataset reported by Hashiguchi and Nishida

(2006). CaSR sequences were used as an out-group. Nucleotide sequences were aligned by codons using the program ClustalW2 inside SeaView5 package (Gouy, Guindon et al. 2010). The eight conserved amino-acid sensing residues were identified in all OlfC sequences and represented using WebLogo application version 2.8.3 (Schneider and Stephens 1990, Crooks, Hon et al. 2004). Using the best-fitting model, GTR+I+G model (determined by jModeltest2 program (Guindin 2011, Darriba D., Taboada G.L. et al. 2012)), a Bayesian phylogenetic tree was constructed with MrBayes software version 3.2.2 (Ronquist and Huelsenbeck 2003). The reliability of our tree was assessed by bootstrap support with total number of 1 000 000 generations. The first 25 percent of the results were discarded for the calculation of the posterior Bayesian probability.

## Results and discussion

### Structure of vomeronasal receptors

VRs are mainly expressed in an anatomically well-defined bony capsule on the anterior nasal septum, the VNO (Yang, Shi et al. 2005) (Figure 3). The vomeronasal system is directly linked with limbic brain structures important in chemical communication (Keverne 2004). Two main super-families of vomeronasal receptors (V1R and V2R) are known with different expression location and gene structure (Grus and Zhang 2004). Both receptors belong to seven-transmembranar G-protein coupled receptors (GPCR) family but while V1Rs with Gαi2-coupled protein are expressed in apical layer of the vomeronasal epithelium and have axonal projections to the anterior accessory olfactory bulb, the V2Rs with Gαo-coupled protein are expressed in the basal layer of the vomeronasal epithelium with axonal projections to the posterior accessory olfactory bulb (Grus and Zhang 2004, Yang, Shi et al. 2005, Young and Trask 2005, Shia and Zhang 2007, Chamero, Leinders-Zufall et al. 2012) (Figure 3).

This kind of dual expression (presence of both Gαi2 and Gαo proteins in neurons of VNO) is referred by segregated type and as several studies showed this feature in rodents (Shinohara, Tokimo et al. 1992, Jia and Halpern 1996, Sugai, Sugitani et al. 1997, Halpern, Jia et al. 1998) and opossum (Halpern, Shapiro et al. 1995), the results were for long time extended to all other vertebrates. However, other types of expression are known, namely the uniform type where neurons only retain the expression of Gαi2 protein (Takigami, Wakabayashi et al. 2004). This last kind of neurons expression has been found in several terrestrial mammals, such as goat (Takigami, Wakabayashi et al.

2004), dog, horse, musk shrew and marmoset (Takigami, Mori et al. 2004) which support a more common expression than previously recognized. Moreover, goats express the V1R and Gαi2 proteins in the main olfactory epithelium (Wakabayashi, Mori et al. 2002). Different pattern of expression also occur in tammar wallaby with intermediate (between uniform and segregate) type of Gαi2 and Gαo proteins expression (Renfree, Papenfuss et al. 2011).

In *Xenopus tropicalis* a different scenario of VRs expression occur with V1R genes being expressed in the main olfactory epithelium (MOE) and not in the vomeronasal organ. As it is believed that VRs firstly appear in amphibians, MOE should had been the first place for V1R expression that managed to retain the expression (e.g. amphibians) or was lost with the more organized vomeronasal organ (e.g. mammals) during vertebrate evolution (Date-Ito, Ohara et al. 2008). In *Xenopus tropicalis* the V2R repertoire also have some elements expressed in MOE, the earlier-diverging genes, whereas others are expressed in VNO, the later-diverging genes (Hagino-Yamagishi, Moriya et al. 2004, Syed, Sansone et al. 2013).

Structurally, V1Rs are much smaller than V2Rs. Considering Vmn1r45 (318 residues length) and Vmn2r26 (855 residues length) from *Mus musculus* we can show the discrepancy in size of both genes. However, the seven transmenbranar domains are present in both sequences and the region that confer difference between receptors in the extent N-terminal region present in V2R gene. At genomic level, Vmn1r45 is entirely encoded by a single exon while Vmn2r26 is encoded by six exons (Figure 3).

At functional level, V1Rs are reported to be associated with the detection of small volatile molecules involved in the gender discrimination and sexual behaviors, whereas V2Rs are involved in detection of water-soluble peptides and control of pheromone-induced male-male aggression (Grus and Zhang 2004, Yang, Shi et al. 2005, Young and Trask 2005, Shi and Zhang 2007, Chamero, Leinders-Zufall et al. 2012).

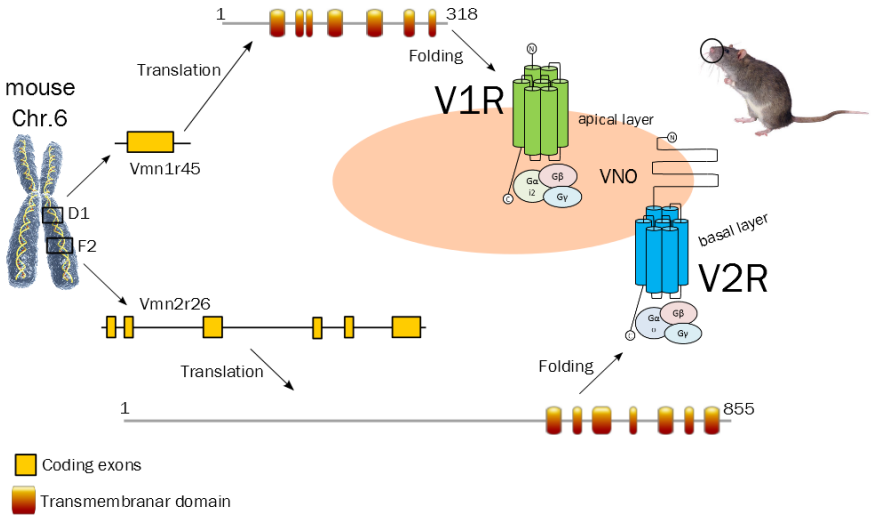



Figure 3 – Example of two V1R and V2R mouse genes expression and structure. V1Rs are encoded by single exons and has small size when compared with V2Rs that are encoded by six exons and possesses large N-terminal region. Both genes are expressed in VNO located in nasal cavity but V1R is located in apical layer whereas V2R in basal layer.

## Water-land transition driving VR evolution: facts and contradictions.

As V1R receptors are more commonly related with the detection of small volatile molecules and V2R receptors are responsible for the detection of water-soluble peptides, it was suggested that during the early tetrapods transition from water to land the V1R repertoire was originated and expanded to provide an efficiently way to detected air-bone ligands, whereas the V2R repertoire would have contracted (Shi and Zhang 2007). We investigated the available number of V1R and V2R genes across a huge number of vertebrates, as also VNO characteristics, in order to understand if repertoire discrepancies are related with environmental transitions.

In rodents it was reported an extensive V1R repertoire (Figure 4b) that is responsible for crucial functions (Boschat, Pelofi et al. 2002) such as the recognition of urinary volatile and sulphated steroids (Liberles 2013). If a cluster of V1R genes becomes lost in mouse, it will occur modifications in behavioural responses such as reduced male libido or inappropriate maternal aggressive behaviour (Emes, Beatson et al. 2004). However, the involvement of V1R in sensory and reproductive functions could explain the high size variation of mammalian V1R repertoires (Grus, Shi et al. 2005). Rodents also show a great repertoire of V2R genes, organized in genomic clusters that have an important role in detect several peptides ligands of MHC class I molecules (Leinders-Zufall, Brennan et al. 2004). However, the number of V2R genes is lower comparatively with the number of V1R elements (Young and Trask 2005, Dong, Jim et al. 2012).

The discrepancy between the number of V1R and V2R genes is visible in other mammals (Figure 4b). In primates, the V1R repertoire is correlated with anatomical development of VNO and strepsirrhinis like bushbabies and mouse lemurs have a well-developed VNO and also a high V1R repertoire (Hohenbrink, Radespiel et al. 2012). In the platyrrhini marmoset it has been documented an important role of VNO in social behaviours such as recognition of group members and reproductive status (Gioigi and Rouquier 2002) and, associated to a medium-size VNO, it was described a small V1R repertoire (Young, Massa et al. 2010). However, relatively to the V2R repertoire, primates were known to not have functional V2R genes (Wakabayashi, Mori et al. 2002, Young and Trask 2005, Shi and Zhang 2007, Ohara, Nikaido et al. 2009, Dong, Jin et al. 2012). Surprisingly, in 2013 appeared the first evidence of two putatively functional V2R genes in platyrrhines primates such as marmoset (Hohenbrink, Mundy et al. 2013), which belong to the New World monkeys group, characterized by having a putatively functional TRPC2 gene (see section 3.4) (Shi and Zhang 2007). Despite the two identified genes being conserved, positive selection was detected in some codons supporting the hypothesis that intact V2Rs in platyrrhines still have an important role in pheromone detection but only expression data in the VNO tissue will fully clarify the relevancy and function of these receptors (Hohenbrink, Mundy et al. 2013).

In ruminants, cow have a well-developed VNO (Salazar, Sanchez-Quinteiro et al. 2008) and express approximately 40 V1R genes (Saraiva and Korsching 2007, Young, Massa et al. 2010, Brykczynska, Tzika et al. 2013) (Figure 4b). In goat and sheep, despite lacking yet an available complete genome sequencing, there are known 23 and 21 cow-similar V1R genes, respectively, but with a complete genome assembly the real number of genes could increase and be similar to those found in cow (Ohara, Nikaido et al. 2009). The importance of pheromonal communication in ruminants is supported by the fact that all goat and sheep V1R genes have orthologs with the same family distribution in cross-species ruminant counterparts, which may suggest that: a) ruminant V1Rs could detect the same/closely related chemical compounds, and b) might detect compounds that are evolutionary conserved or essential for the survival of these species like the detection of male effect pheromones (Ohara, Nikaido et al. 2009). However, no V2R genes were found in ruminants, apart from a few pseudogenes (Young and Trask 2005, Ohara, Nikaido et al. 2009, Dong, Jin et al. 2012).

Even in more basal mammalian species, like marsupials and monotremes, some differences exist in the ratio of V1R:V2R gene numbers (Figure 4b). High repertoires, with above 90 V1R genes were reported in grey short-tailed opossum and tammar wallaby (Shi and Zhang 2007, Young, Massa et al. 2010, Dong, Jin et al. 2012), that have well-developed VNO (Schneider, Fletcher et al. 2009, Schneider 2011). Similar to

marsupials, the monotreme platypus has a high number of V1R genes (~280 genes) (Young, Massa et al. 2012). These marsupials and monotremes V1R genes form monophyletic groups that arose by duplication, which suggest adaptation in order to have a profitable pheromonal communication system (Goodstadt, Heger et al. 2007, Grus, Shi et al. 2007, Warren, Hillier et al. 2008, Schneider 2011) that could be related with the dependence of shortly reach of milk source after birth or hatching (Schneider, Fletcher et al. 2008, Schneider 2011). In opossum, 86 V2R genes were reported (a little less than the number of V1R genes) (Young and Trask 2005, Shi and Zhang 2007) but in platypus only 15 V2R genes were reported (Dong, Jin et al. 2012, Brykczynska, Tzika et al. 2013).

Within amphibian species, an opposite scenario occurs with high number of V2R genes, comparatively to the V1R genes (Figure 4b). For example, the red-legged salamander (*Plethodon shermani*) have highly dynamic VNO, with number of cellular division epithelial layer varies seasonally leading to alterations in the volume of the organ (Dawley, Fingerlin et al. 2000), associated with the expression of 34 V2R genes (Kiemnex-Tyburczy, Woodley et al. 2012). The ligands of these receptors are still unknown but salamanders use chemical cues in social and reproductive interactions (Park, McGuire et al. 2004, Kiemnec-Tyburczy, Woodley et al. 2012) and it was hypothesized that V2R can also have a relevant function in summer foraging (Woodley 2010). Difficulties in V1R amplification have occurred making difficult to precisely the real size of the V1R repertoire, which is believed to be small (Kiemnec-Tyburczy, Woodley et al. 2012). . Frogs are well adapted to both terrestrial and aquatic environments (Gliem, Syed et al. 2013), making possible the use of both water-soluble and volatile chemicals as pheromones (Date-Ito, Ohara et al. 2008). In *Xenopus tropicalis* there were found more than 330 V2Rs, suggesting gene expansion (Ji, Zhang et al. 2009) and highlighting the importance of pheromonal communication (Woodley 2010). However, no volatile chemicals have been identified in frog and only 21 putatively V1R genes were identified in *Xenopus tropicalis* (Shi and Zhang 2007, Date-Ito, Ohara et al. 2008, Brykczynska, Tzika et al. 2013).

Some of the previously examples gave strength to the hypothesis of VR evolution being driving by the water to land transition of ancestral species, but contradictory facts in squamate reptiles are appearing. Snakes use their tongue to collect environmental chemical cues (Schwenk 1995, Filoramo and Schwenk 2009) that are conducted by a well-developed vomeronasal organ without seasonally variations (Takimi 2002, Rehorek, Firth et al. 2009, Saito, Oikawa et al. 2010, Kondoh, Yamamoto et al. 2012).

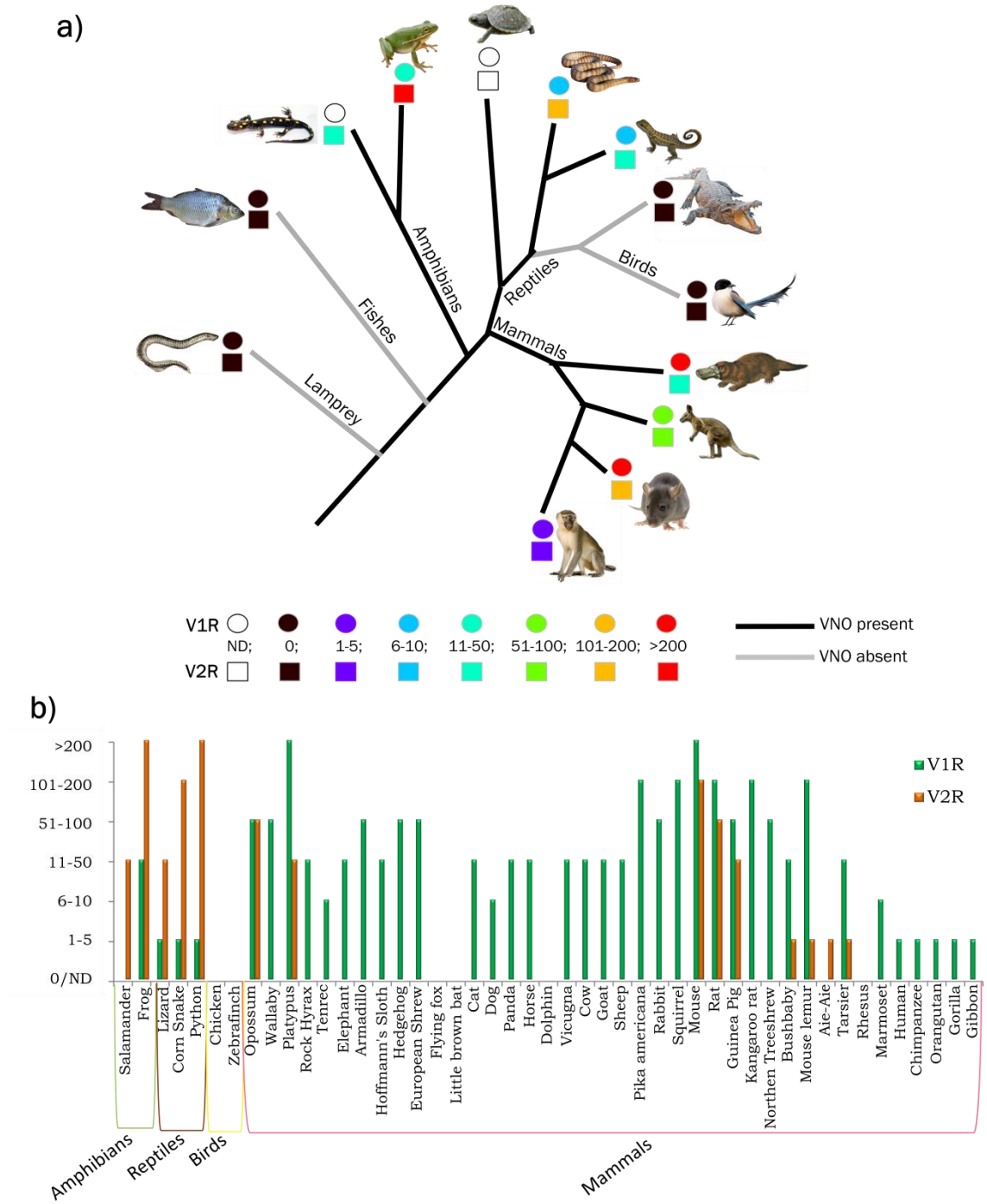



Figure 4 - Distribution of vomeronasal organ (VNO) and vomeronasal receptors (VRs) among vertebrates. a) The presence of VNO (black branches) is connected with presence of V1R and/or V2R (circles and squares, respectively) with the exception of turtle where, despite the presence of VNO, the information about VRs is not available. The color spectrum of circles and squares is connected with repertoire size of V1Rs and V2Rs, respectively: purple, 1 to 5 genes; blue, 6 to 10 genes; little blue, 11 to 50 genes; green, 51 to 100 genes; orange, 101 to 200 genes; red, more than 200 genes; white, no data available. In species that lose VNO (grey branches) it has not been identified VRs, which suggest a strong relationship between the presence of VNO and VRs. b) Variations in V1R and V2R repertoire number among vertebrates (Young and Trask 2005, Shi and Zhang 2007, Ohara, Nikaido et al. 2009, Young, Massa et al. 2010, Dong, Jin et al. 2012, Brykczynska, Tzika et al. 2013) are represented by green bars (number of V1R genes) and orange bars (number of V2R genes). Non-mammalian species usually have more V2R than V1R genes whereas in mammals V1R genes are often more common.

Despite it was found large V2R repertoires (almost 109 and 216 genes in *Phantherophis guttatus* and *Python molurus bivittatus*, respectively*),* only a few V1R genes were identified (Figure 4b) which could be explained by a) an ancestral small V1R repertoire that did not expanded in squamates or b) a large V1R repertoire that would have contracted and resulted in a few remnants today (Brykczynska, Tzika et al. 2013). As the squamates V1R repertoire is not expanded, the hypothesis loses credibility (Brykczynska, Tzika et al. 2013). However, more studies are needed, mainly in reptiles, to understand how VR genes are evolving and which factors drove the expansion or contraction of those receptors among vertebrate species.

Dog domestication and vomeronasal repertoire contraction.

In carnivores, while dog have an organized VNO with all characteristic elements (Dennis, Allgier et al. 2003), no V2R genes and only a small V1R repertoire with less than 10 genes were reported (Grus, Shi et al. 2005, Young and Trask 2005, Shi and Zhang 2007, Young, Massa et al. 2010, Dong, Jin et al. 2012), which is surprisingly due to high social and specific interactions among dogs (Quignon, Rimbault et al. 2012).

It was reported that the wolf/dog split might be coincident with dog domestication (Arnason, Gullberg et al. 2007) and it was hypothesized that domestication had been responsible for deterioration of V1R dog genes. However, a recent study showed that wolf has the same inactivating genes as dog, which make unlikely that domestication drove the V1R loss in dog (Young, Massa et al. 2010) and other factors should be responsible for the V1R contraction. On the other hand, the cat that also was under domestication, have a well-developed VNO (Salazar and Sanchez-Quinteiro 2011) and medium size V1R repertoire (28 elements) (Young, Massa et al. 2010). Cow and sheep, altought not carnivores, are two other species under domestication by humans since ancient times. However, similarly to cat, cow and sheep present a medium size V1R genes repertoire, which withdraw strength to the hypothesis that domestication influenced the V1R repertoire contraction in dog.

TRPC2 gene and vomeronasal communication

Trpc2 gene codifies a transient receptor potential channel 2 that has been reported as crucial in neuronal signaling in the vomeronasal organ and it is believed that TRPC2 channel is responsible for $Ca^{2+}$ intake step in vomeronasal communication (Yildirim and Birnbaumer 2007, Young, Massa et al. 2010). In case of male Trpc2

knockout mice, studies refer that they have difficulties in discriminate males from females and lose the natural attack behavior with other males (Halpern 2003).

Given the importance of TRPC2 channel in vomeronasal communication, we used the Ensembl database and the Genomicus browser to analyze the distribution and synteny of TRPC2 gene in vertebrates (Figure 5).

We found a widespread distribution of TRPC2 gene across mammals, reptiles, amphibians and fishes. TRPC2 in mammals and reptiles presents a well-conserved synteny with NUMA1, IL18BP and Rnf121 genes flanking its tail region and Art5 and Art1 genes flanking the head region (Figure 5a, 5c). In fishes, the tail flanking genes present in tetrapods are not conserved and instead of Art5 and Art1 genes, the head region presents a conserved RRM1 gene (Figure 5e). TRPC2 gene in *Xenopus tropicalis* genome is flanked by LRF1 gene and other uncharacterized genes, but since only this amphibian genome is currently available, we cannot conclude about the conservation of its synteny across amphibian species.

Some studies reported that pseudogenization of TRPC2 in birds and Old World Monkeys are usually connected with vomeronasal communication absence (Grus and Zhang 2006, Shi and Zhang 2007). We explored the presence/absence of TRPC2 gene in avian species, but we did not found such genes in the five bird genomes analyzed (Figure 5b).

Within primates, humans have a TRPC2 pseudogene (Figure 5a.1), but despite the presence of some V1R genes there are currently doubts about their real functionality. In orangutan and rhesus macaque, which are old world monkeys, we detected three copies of the TRPC2 gene (Figure 5a.1), which could be related with the additional role that this channel have in the induction of acrosomal reaction during the fertilization process (Yildirim and Birnbaumer 2007).

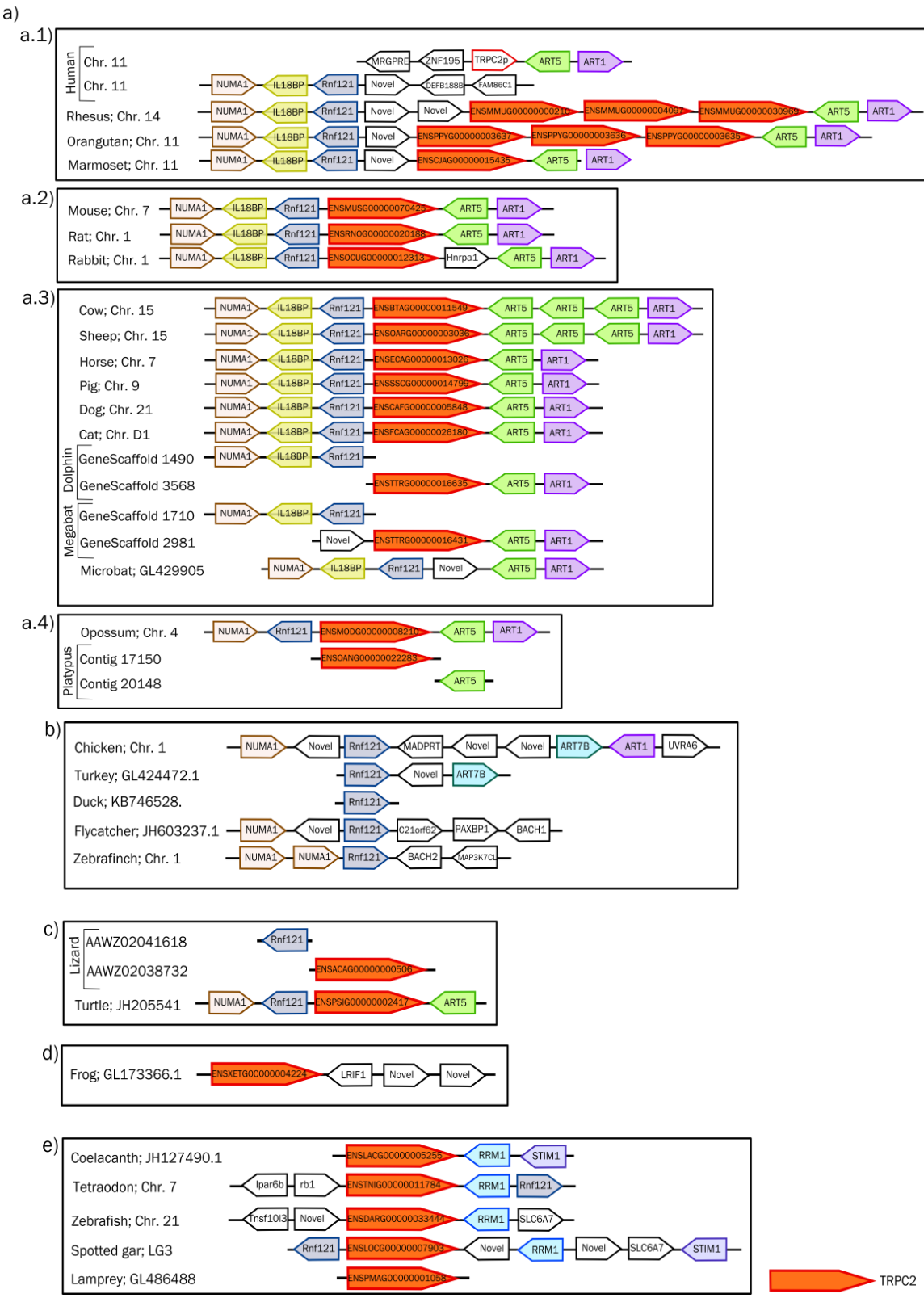



Figure 5 – TRPC2 gene synteny across vertebrates. TRPC2 genes were detected in mammals, amphibians, reptiles and fishes. Mammals and reptiles show a conserved synteny of the flanking genes. Fishes, with exception of lamprey, only share one flanking gene with mammals. In birds, no TRPC2 genes were detected.

Selective pressure in the evolution of vomeronasal receptors.

One of the most common ways to evaluate how genes are evolving includes the characterization of the proportion of sites with non-synonymous substitutions (Ka) relatively to sites with synonymous substitutions (Ks), the Ka/Ks ratio. Ka/Ks ratio lower than 1 indicates that the protein is under purifying pressure which means that there are a selection against changes in the protein sequence to conserve it. When the Ka/Ks ratio is higher than 1, the protein is under positive selection favouring the retention of advantageous mutations to improve protein function (Mouse Genome Sequencing Consortium, Waterston RH et al. 2002).

Selection studies on mouse and vomeronasal receptors detected some sites under positive selection but with Ka/Ks ratio very close to 1 (about 5-10% of the codons) (Zhang, Rodriguez et al. 2004). V1R genes were considered to be under the influence of positive Darwinian selection (Emes, Beatson et al. 2004, Lane, Young et al. 2004). It was also hypothesised that if in one hand, the positive selection pressure may maintain functional genes together in the genome in order to organize regulatory domains (Zhang and Webb 2003), in the other hand if a mixture of pheromones are evolving by a rapid neutral process this situation could create a strong selection pressure in recognition systems, like V1R, to quickly adapt to pheromone alterations (Shi, Bielawski et al. 2005). However, in all of these studies it was used paralogous sequences, which make unreliable the inference of positive selection events between orthologous (Grus and Zhang 2004). More recently it was proposed that despite occasional actions of positive selection, the evolution of rodent V1Rs is in large part influenced by purifying selection and random drift (Park, Podlaha et al. 2011). Relatively to V2R genes, the study of a small number of genes in rodents revealed some codons under positive selection mainly in extracellular domains (Emes, Beatson et al. 2004). Further studies suggested that they only have a relatively weak purifying selection and/or positive selection acting in the N-terminal region of rodents V2Rs, which is thought to be the ligand-binding domain (Yang, Shi et al. 2005). Regarding other species, only in the mouse lemur were identified several residues under positive selection in V1R genes (Hohenbrink, Radspiel et al. 2012). The analysis of strepshirrhines V2Rs genes identified only one gene with some codons under positive selection (Hohenbrink, Mundy et al. 2013).

Both V1R and V2R genes lack evidence of strong positive selection driving their evolution. However, the fact that previous studies have only been conducted in a limited number of taxa does not allow generalizing such considerations. Thus, further studies with other species, mainly non-mammalian, and testing other statistical methods will be useful to understand the evolution of genes involved in pheromonal communication.

Loss of the Vomeronasal organ *versus* loss of vomeronasal communication.

Vomeronasal system is present in many vertebrates but there are species, punctual taxa or large families, where the vomeronasal organ, and the consequently vomeronasal communication were not still detected or is believed to be absent (Figure 4a). Several studies have tried to understand which factors and reasons drove such distribution. We explored several species which do not have available information about vomeronasal receptors repertoire, to understand if vomeronasal system is in fact absent and if there is a close relationship between presence of VNO and vomeronasal receptors.

One of the best representative examples of total absence of vomeronasal system includes birds. In chicken, the VNO was not reported (Døving and Trotier 1998) and V1R and V2R genes or pseudogenes were not found. Our Ensembl database search did not reveal vomeronasal receptor proteins in the six bird genomes available (budgerigar, chicken, turkey, duck, zebra finch and flycatcher). We also conducted searches in the NCBI GenBank database using the Blast search to identify vomeronasal genes inside birds. In all bird genomes analysed Blast searches found punctual match sequences but all of them have low score identity (less than 50%) and very low query cover (less than 10%). In addition, Blast search of these punctual match sequences do not have vomeronasal receptors as best hit. The present scenario could be justified by the fact that the detected proteins could be related with GPCR family elements, sharing some similarity, but these results were discarded as belonguing to vomeronasal receptors.

TRPC2 genes or pseudogenes were also not detected in chicken or in other birds (Figure 4b). Some authors had considered that birds do not have VNO-communication and even the pseudogenization of genes involved in transduction pathways took place long ago making them unidentifiable in the chicken or the zebrafinch genomes (Shi and Zhang 2007, Dong, Jin et al. 2012). It is known that birds are not anosmic, as it had been considered for long time, and chemical odorants are important for orientation, food location or nest location but no pheromones or evidence of pheromonal communication have been detected to date in birds (Caro and Balthazart 2010). The fact that birds have an excellent visual and acoustic acuity (more important features for flying behaviour than for olfaction) (Caro and Balthazart 2010) and highly marked sexual dimorphism (related with reproductive activity), could explain the vomeronasal system degeneration in birds.

It was also suggested that archossaurs, the common ancestors of birds, crocodilians and dinosaurs, also do not had VNO (Taniguchi, Saito et al. 2011) and until

now no information was reported about crocodilian vomeronasal receptors (Døving and Trotier 1998, Mason and Parker 2010, Saito, Oikawa et al. 2010).

However, our searches in the NCBI database revealead six Predicted Vomeronasal-like sequences annotated in crocodilians (XM_006031313.1, XM_005291513.1, XM_006020910.1) and turtles species (XM_005303611.2, XM_007059608.1, XM_006273626.1). Our previously analyses revealed that all chemosensory receptors, including VRs have seven transmembranar domains. In addition, V1R, T2Rs, Taars and ORs show a conserved G protein family 1 profile (also present in other GPCRs), characteristic by having an acidic-Arginine-aromatic triplet in the N-terminal extremity of the second cytoplasmic loop that could be implicated in the interaction with G proteins (Strosberg 1991). We searched for these domains and motifs in order to confirm the identity of the putative sequences. While all sequences presented a conserved G protein profile family 1, the 7 transmembranar domains were not found in the annotated V1R3-like sequences of Chrysemys *picta bellii* (XM_005303611.2, XM_005291513.1) and *Chelonia mydas* (XM_007059608.1). *Alligator sinensis* V2R-like and V1R1-like sequences (*XM_006020910.1* and XM_006031313.1, respectively) present the seven characteristic transmenbranar domains but blast searches failed to hit putative "vomeronasal-like" sequences with previously annotated vomeronasal receptors, being the best hit sequences of other GPCR family elements, such as taste receptors or galanin receptors (Figure 6). We suggested that the reported reptilian sequences are members of GPCR family but do not seem members of the vomeronasal receptors subfamily. Currently data lead us to infer that vomeronasal receptors are not present in crocodilian and turtle genomes, which is in accordance with previously references (Døving and Trotier 1998, mason and Parker 1998, Saito, Oikawa et al. 2010), but further well-coverage genomes and new analytical tools could change the currently scenario.

**a)** **b)**

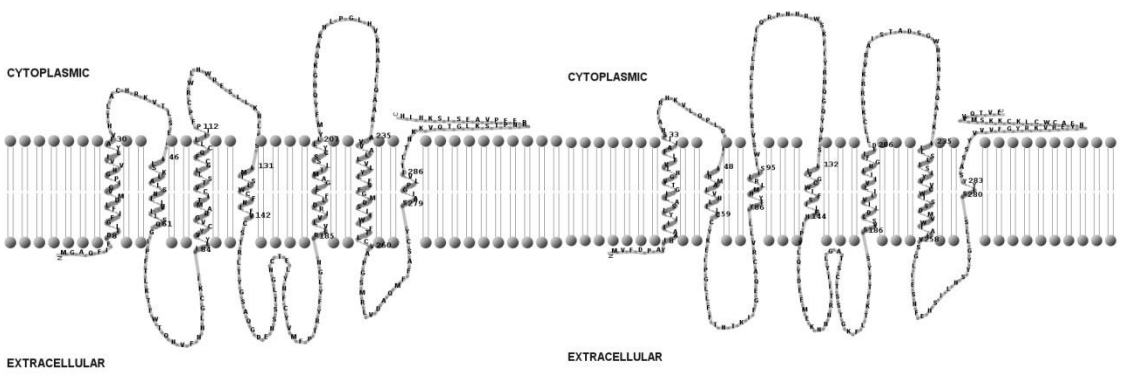



Figure 6 – Putative *Alligator sinensis* chemosensory receptors. Putative V1R1-like sequence (a) (ID: XM_6031313.1) and putative V1R-2L sequence (b) (ID: XM_003020910.1) present seven transmembranar domains and conserved G protein family 1 profile but in Blast search failed to match with vomeronasal receptors and went discarded.

In Hominids and Old World Monkeys, the VNO was lost or is a very rudimentary one (Smith, Garrett et al. 2011) and are associated with absent or small V1R repertoires (Kambere and Lane 2007, Young, Massa et al. 2010), which suggest the reduced use of chemicals to communicate (Giorgi and Rouquier 2002). In addition, the decline of pheromonal communication in catarrhinis is coincident with the evolution of trichromatic colour vision and dominance of the primate visual system (Zhang and Webb 2003).

In Bats, a developed VNO was only reported in *Miniopterus*, *Pteronotus* and phyllostomid bats (Bhatnager and Meisami 1998) being rudimentary or absent in all other bat species (Wible and Bhatnagar 1996, Bhatnagar and Meisami 1998). Flying fox and Little brown bat, do not have VNO (Bhatnagar and Meisami 1998) and it was suggested that they lost their V1R genes (Zhao, Xu et al. 2011, Jones, Teeling et al. 2013). Previously studies reported that bats only possess TRPC2 pseudogenes (Zhao, Xu et al. 2011, Jones, Teeling et al. 2013). However, in our study we detected one syntenic TRPC2 gene in the Ensembl database for the Flying fox bat (Megabat) (Figure 5a.3), which was not found in the Microbat genome. Since there is no more available bat genomes in Ensembl database, our search were limited to this duality in bats.

Nevertheless, we searched V1R genes in bats genomes but WGS searches do not conducted to reliable results. In addition, we found four sequences in NCBI database annotated as vomeronasal-like receptors. Putative V1R-90 like sequence of *Pteropus alecto* (XM_006917652.1) and putative V1R5a and V1R4-like sequences of *Eptesicus fuscus* (XM_008158126.1 and XM_008154120.1, respectively) have a short size, absence of conserved domain and a reduced number of transmembranar domains. It was also identified a short V1R2-like sequence of *Eptesicus fuscus* (XM_008141256.1) that have a premature stop codon. Due to structural differences, but considering that the four bat sequences blast with other mammalian vomeronasal sequences, these sequences could correspond to a) incomplete sequences resulting from sequencing artefacts or b) vomeronasal pseudogenes (based on short size and presence of stop codons) but with the current information, they should not be considered functional vomeronasal receptors. It was suggested by other authors that the vomeronasal “loss-of-function” in bats do not appear be related with sensory-trade off since this phenomenon is widespread in echolocation and non-echolocation taxa, in dichromatic and monochromatic bats (Zhao, Xu et al. 2011).

Aquatic mammals are other group where it was reported that chemical communication should be unimportant, with absence of the vomeronasal system in cetaceans such as dolphins (Oelschlager 1989, Swaney and Keverne 2009). Studies conducted by other authors do not found any vomeronasal receptor (Oelschlager 1992,

Meisami and Bhatnagar 1998, Young, Massa et al. 2010) but due to our finding of TRPC2 gene in the dolphin genome (Figure 5a.3), we searched the presence of vomeronasal receptors in cetaceans. Seven putative cetacean sequences, which blast with other mammalian vomeronasal sequences, were found in NCBI database, identified as vomeronasal like sequences. Analysing the structure and presence of conserved domains, partial V1R1-like and V1R3-like of *Physeter catodon* (XM_007121851.1 and XM_007119963.1, respectively) and partial V1R-4 like sequence of *Lipotes vexillifer* (XM_007451374.1) do not present conserved domains, neither transmembranar regions, but due to their very reduced size we cannot conclude about the real identity of these sequences. *Baleanoptera acutorostrata scammoni* sequences (V1R2-like - GI:594663674 and V1R1-like - GI:594663585) and *Tursiops truncates* sequence (V1R1-like - XM_004313804) present the conserved domain of G protein receptor profile 1 but do not have seven transmembranar domains. Attending to these features, we suggest that the putative sequences do not correspond to complete vomeronasal receptors but we are not able, with the current tools available, to support their identity.

A different scenario occurs with another V1R-4 like sequence of *Lipotes vexillifer* (XM_007459344.1). This gene blasted with other mammalian vomeronasal receptors, have the conserved G protein family 1 profile and possess seven transmenbranar domains (Figure 7). According with this information we suggest that *Lipotes vexillifer* possesses a sequence that shares common features with mammalian vomeronasal receptors. However, due to trace amine-associated receptors, olfactory receptors, and taste receptors, which also present seven transmembranar domains and G protein family 1 profile, we cannot ensure with high confidence the real identity of this sequence.

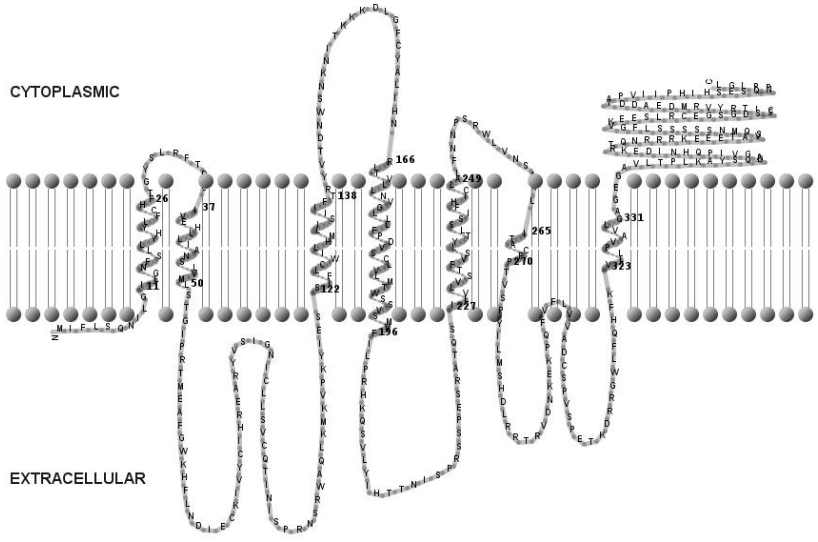



Figure 7 – Structure of putative V1R-like sequence of *Lipotes vexillifer*.

With these analyses we can conclude that doubts remain about the presence or absence of Vomeronasal receptors in some mammals, reptiles and birds, which cannot be ensure with confidence, based on the current available methods. However, our analyses did not detect any signal of vomeronasal receptors in birds, which is supported by references of lack of this system in avian species. In other species, currently results support that vomeronasal system in higher vertebrates is functional where both VNO and VRs are present, but this scenario could be changed if a new strategy to distinguish chemosensory receptors would be used. Moreover, the presence of TRPC2 is not indicative of the presence of vomeronasal communication *per si*, due to other functions of this gene, but in species that detect pheromones by CRs, TRPC2 needs to be present.

Pheromones detection in the absence of VNO.

Communication by pheromones is an old way to transmit information found in basal vertebrates as the sea lamprey, which have sexual (Li 2005) and migratory pheromones (Fine and Sorensen 2008, Cummins and Bowie 2010). In fishes, pheromones such as L-Kynurenine were reported in the urine of female salmon and trout, with important role in male attraction (Cummins and Bowie 2012). However, only tetrapods show an organized vomeronasal system for pheromonal detection. The question is how other vertebrates, like fishes or lamprey are detecting pheromones if they do not have an organized vomeronasal system, which is crucial for pheromones detection? Could they have vomeronasal receptors, independent of the presence of an organized organ to detect pheromones?

Vomeronasal receptors were firstly detected in rodents and used as models to search similar receptors in other taxa (Dulac and Axel 1995). Teleost fishes showed some receptors similar to mammalian receptors, identified as V1R-like and V2R-like receptors (Hashiguchi and Nishida 2005, Pfister and Rodriguez 2005, Hashiguchi and Nishida 2006, Pfister, Randall at al. 2007, Shi and Zhang 2007, Grus and Zhang 2009, Dong, Jin et al. 2012. In addition, TRPC2 gene is present in lamprey and other evolved fishes (Figure 5e), suggesting that VR-like receptors should have similar activation pathways. Recent studies clarified that these genes, renamed as ORAs (olfactory receptors related to class A GPCRs) (Saraiva and Korsching 2007) and OlfCs (Olfactory receptor related to class C GPCRs) (Alioto and Ngai 2006), form monophyletic groups and constitute independent families separated from V1R and V2R families (Hashiguchi and Nishida 2005, Hashiguchi and Nishida 2006, Saraiva and Korsching 2007). Fishes do not have vomeronasal organ (Hashiguchi and Nishida 2005, Hashiguchi and Nishida

2006, Saraiva and Korsching 2007) and only in the more evolved lungfishes, it was detected positive Goα and Giα2 expression in cells of lamellar epithelium (Gonzalez, Morona et al. 2010), which is considered by some authors as a “primordial vomeronasal organ” (Nakamuta, Nakamuta et al. 2012).

In contrast to high developed vertebrates, fish genes responsible for pheromone detection are expressed in a pseudo-stratified olfactory epithelium named olfactory rosette (Pfister and Rodriguez 2005, Pfister, Randall et al. 2007, Saraiva and Korshing 2007, Bazaes and Schmachtenberg 2012, Ota, Nikaido et al. 2012). Therefore, given also the evidence retrieved from fishes, we suggest a strong connection between the non-existence of vomeronasal organ and the absence vomeronasal receptors. In addition, as the primordial VNO is reported in amphibians (Eisthen 1992), the first VRs should have appeared in such species and not in fishes as previously suggested (Hashiguchi and Nishida 2005, Pfister and Rodriguez 2005, Hasiguchia nd Nishida 2006, Pfister, Randall et al. 2007, Shi and Zhang 2007, Grus and Zhang 2009, Dong, Jin et al. 2012).

Similar to teleostei fishes, sea lamprey (*Petromyzon marinus*) do not have organized vomeronasal organ but similar features in nasal epithelium are shared with developing tetrapods (Chang, Chung-Davidson et al. 2013). A few lamprey V1R-like genes were also reported to express in the olfactory epithelium (Laframboise, Ren et al. 2007), which structure and size are similar to V1R (Grus and Zhang 2009, Libants, Carr et al. 2009). We found only one gene and, based on synteny and phylogenetic position, we considered it as ORA3 (Figure 9, Figure 10). Nevertheless, deeper studies should be conducted in future to evaluate if other ORA genes are present in lamprey. No V2R-like genes were yet detected in lamprey which could be due to (1) lack of available full coverage of the genome (currently between 5.9 and 9.3X depending on the estimated genome size) or (2) real absence of these genes in the lamprey genome. The last hypothesis is more plausible and it is possible that V2R-like genes only had appeared after the separation of jawed and jawless vertebrates, because no V2R-like genes have been identified in urochordates (Kamesh, Aradhyam et al. 2008) and cephalocordathes (Nordstrom, Fredriksson et al. 2008). However, more studies are needed in this basal vertebrate to fully determine the total number of elements involved in pheromones detection.

Fishes strategy to detect pheromones.

As it was previously reported, pheromones in fishes are detected by ORAs (Saraiva and Korsching 2007) and OlfCs (Alioto and Ngai 2006) that have different features from the tetrapod receptors.

Ora genes are different from VRs, since the common processes of duplication or pseudogenization associated to VRs were not detected in these fish genes (Pfister, Randall et al. 2007, Saraiva and Korsching 2007). In addition, Ora genes present high degree of homology (Pfister and Rodriguez 2005, Johnson and Banks 2011, Ota, Nikaido et al. 2012), conserved 5'UTR region (Pfister, Randall et al. 2007) and conserved glycosylation site at extracellular loop 2 (Pfister and Rodriguez 2005, Pfister, Randall et al. 2007).

Six classes of ORA genes were reported in teleost fishes that can be grouped into three well defining pairs, Ora1-Ora2, Ora3-Ora4 and Ora5-Ora6 (Saraiva and Korsching 2007, Johnstone, Lubieniecki et al. 2012).

We collected sequences from several fish classes (Figure 8), to confirm the phylogenetic relationships and understand the genomic gene synteny. Maximum number of available sequences was firstly retrieved from the Ensembl database. Additionally, the NCBI database was also checked for the species genomes when the genes under study were not available in Ensembl.

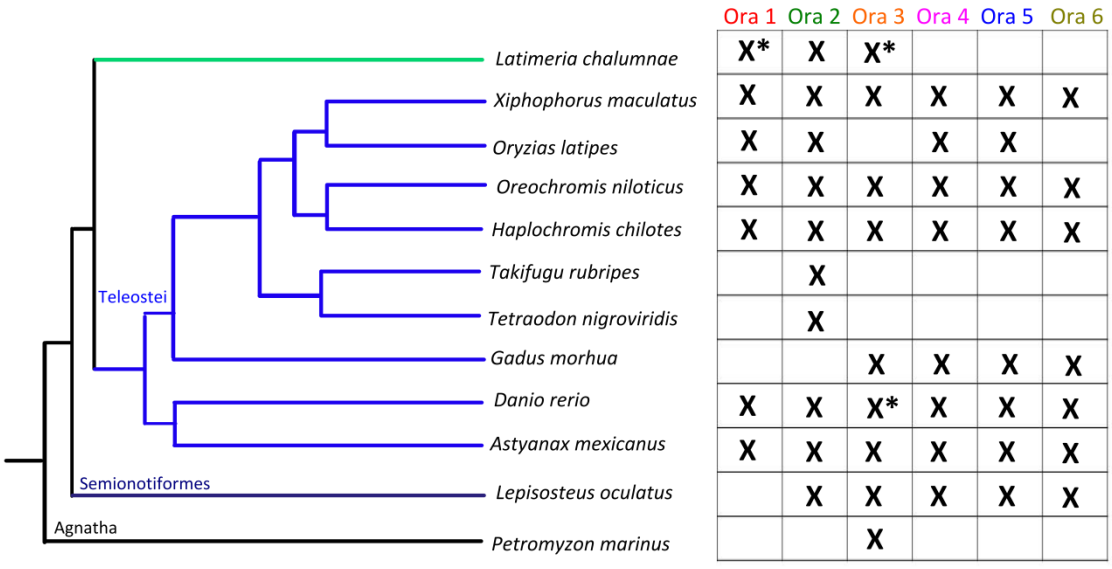



Figure 8 – Phylogenetic relationship between analyzed fishes in this study (Diogo, Doadrio et al. 2008, Near, eytan et al. 2012, Zou, Guo et al. 2012, Opazo, Butts et al. 2013) and distribution of Ora genes. The presence of one Ora gene in a species is represent by a cross. Superscript stars indicate duplication in considering Ora element.

One previous study of Saraiva and Korsching in 2007 showed that the six Ora genes grouped two-by-two in the tree (ora1-ora2, ora3-ora4 and ora5-ora6) suggesting the existence of three ancestral genes (Saraiva and Korsching 2007). Our Bayesian tree (Figure 9), including more sequences than the Saraiva and Korsching (2007), retrieved well-supported branches and suggest a different interpretation with the presence of only two ancestral genes: the Ora5/6 ancestor and the Ora1/2/3 ancestor. After the split, the Ora5/6 ancestor originated Ora5 and Ora 6 genes that are located in different genomic positions (Figure 9 and 10), whereas Ora1/2/3 ancestor originated Ora1/2 ancestor and Ora3 gene. Ora 1 and Ora 2 should have arrived by duplication of Ora1/2 ancestor and Ora4 by duplication of Ora 3 since in both situations the genes are together in the same chromosomic location (Figure 10). The coelacanth Ora 2 gene is the unique gene that phylogenetically did not stayed inside the Ora 2 genes cluster (Figure 9).

It was reported that Ora1-Ora2 and Ora3-Ora4 genes are located closely in the same chromosomic region in head-to-head and tail-to-tail orientation, respectively (Johnstone, Lubieniecki et al. 2012). This result was confirmed by our study. Ora 1 and Ora2 genes were in head-to-head orientation in platyfish, medaka and tilapia and present Irig2 and Padi2 genes flanking the Ora 2 side. This orientation is also present in cavefish and coelacanth but both species do not have conserved flanking genes (Figure 6a).

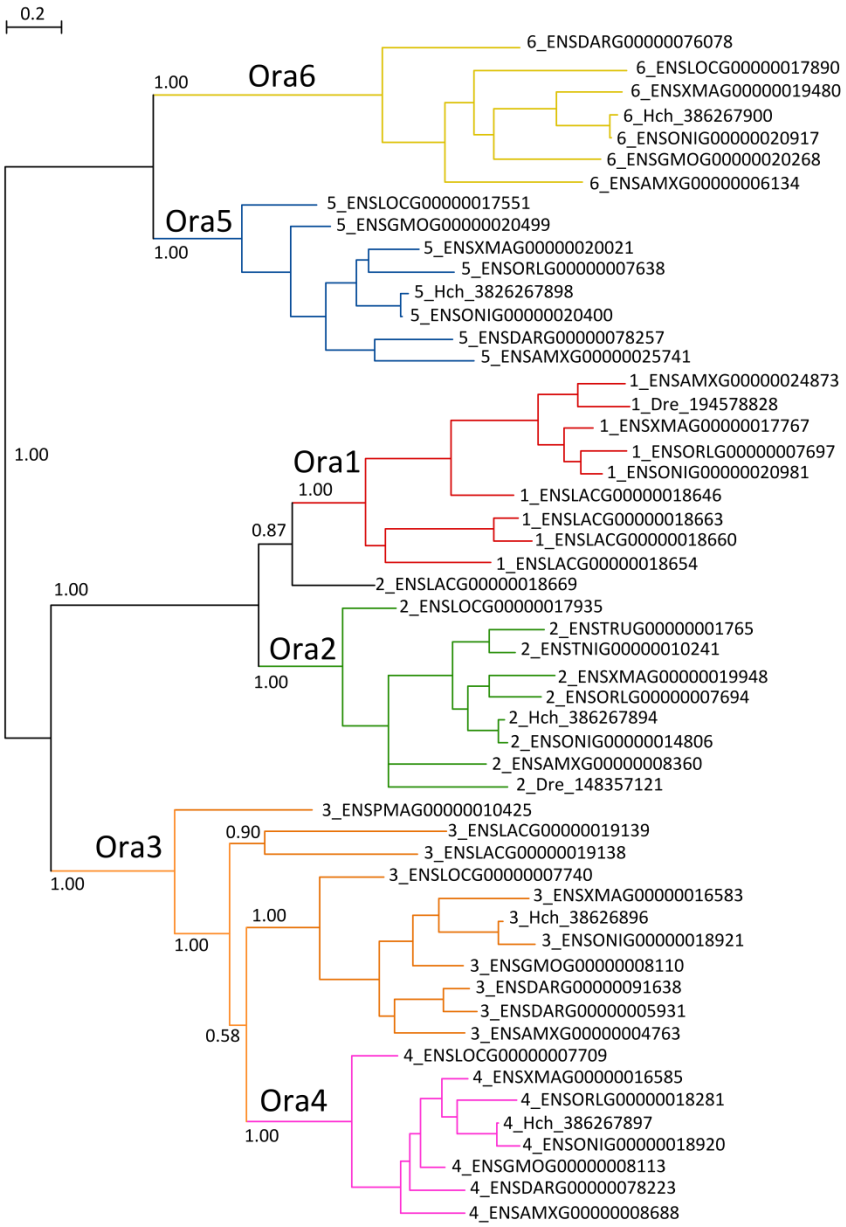


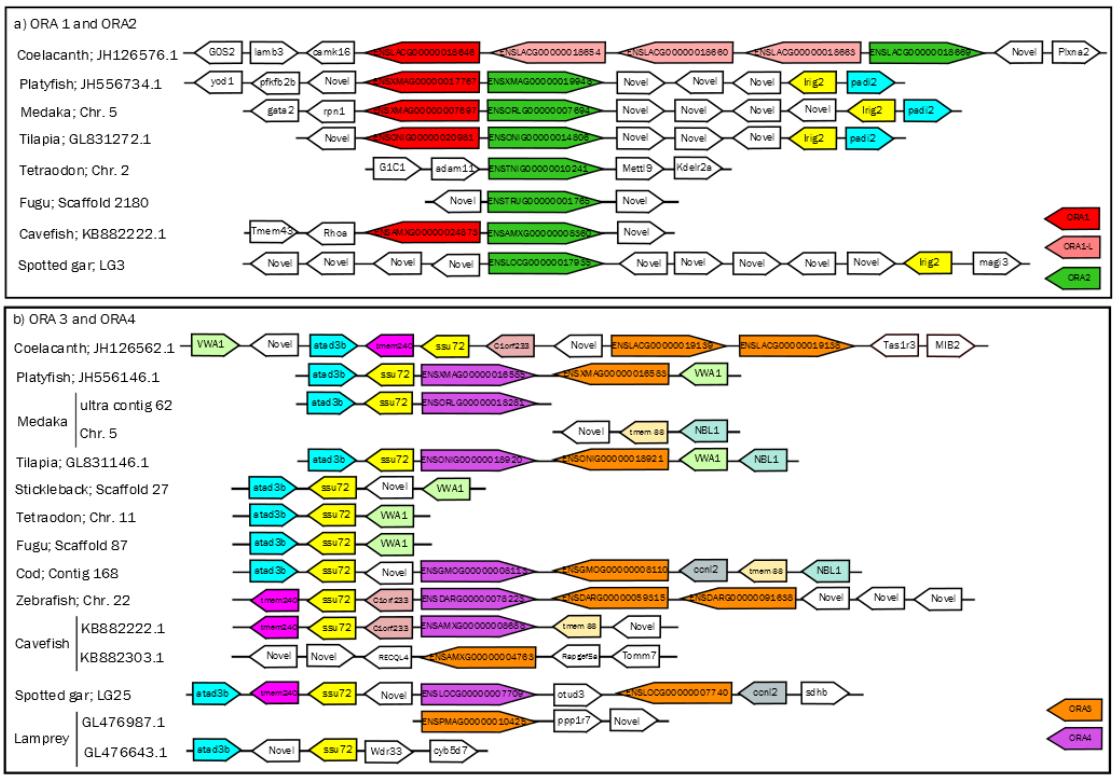

Figure 9 – Phylogeny of the Ora genes. Bayesian tree were constructed under GTR+I+G model and with 385000 bootstrap replications. Trees were stopped when deviation split frequencies were lower than 0.05. Bootstrap values under each branch.

In addition, in the coelacanth genome there are more three Ora 1-like genes that form an isolated group but close to other fishes Ora 1 gene (Figure 10a). Ora1 gene were not detected in tetraodon (green spotted pufferfish), fugu and spotted gar (Figure 8 and 10a). Ora 3 and Ora 4 genes are in close location, in tail-to-tail orientation (with exception of the cavefish that did not present Ora 3 and Ora 4 genes in the same location), and usually flanking by atad3b-Temem240-ssu72 genes in the Ora 4 side. Flanking genes in Ora 3 side are not too conserved. Ora 3 and Ora 4 genes were detected in all fishes with exception of coelacanth and lamprey, where only the Ora 3

was detected, and the medaka, where only the Ora4 gene was detected. In tetraodon and fugu, neither Ora 3 nor Ora 4 genes were detected (Figure 10b).

Despite it was suggested that Ora 5 and Ora 6 genes resulted from the same ancestor, they are not located in same chromosomic region. Ora 5 gene possesses conserved synteny and is located close to nup155 gene. The Ora 5 gene was detected in all species with exception of coelacanth, tetraodon, fugu and lamprey. Surprisingly, these species also do not present Ora 6 gene, which could suggest an ancient lack of Ora5/6 ancestor. Medaka is the unique analyzed species that present Ora5 genes despite the absence of the Ora 6 gene. However, in species that have Ora 6, the gene presents a well-conserved synteny, being flanked by yy1b-evlb genes and MGA-Mapkbp1-pak7 genes.

It was reported a strong negative selection acting in Ora genes, which is drastically different from the scenario present in mammalian V1R family, characterized by fast evolution and subsequently highly species-specific gene repertoires (Saraiva and Korsching 2007, Johnson and Banks 2011). Studies in rockfishes Ora2 gene found significant evidence of positive selection in some sites (Johansson and Banks 2011) but more studies, with more species should be conducted to confirm it. The function of Ora genes and also their ligands are currently unknown (Saraiva and Korsching 2007, Johansson and Banks 2011).



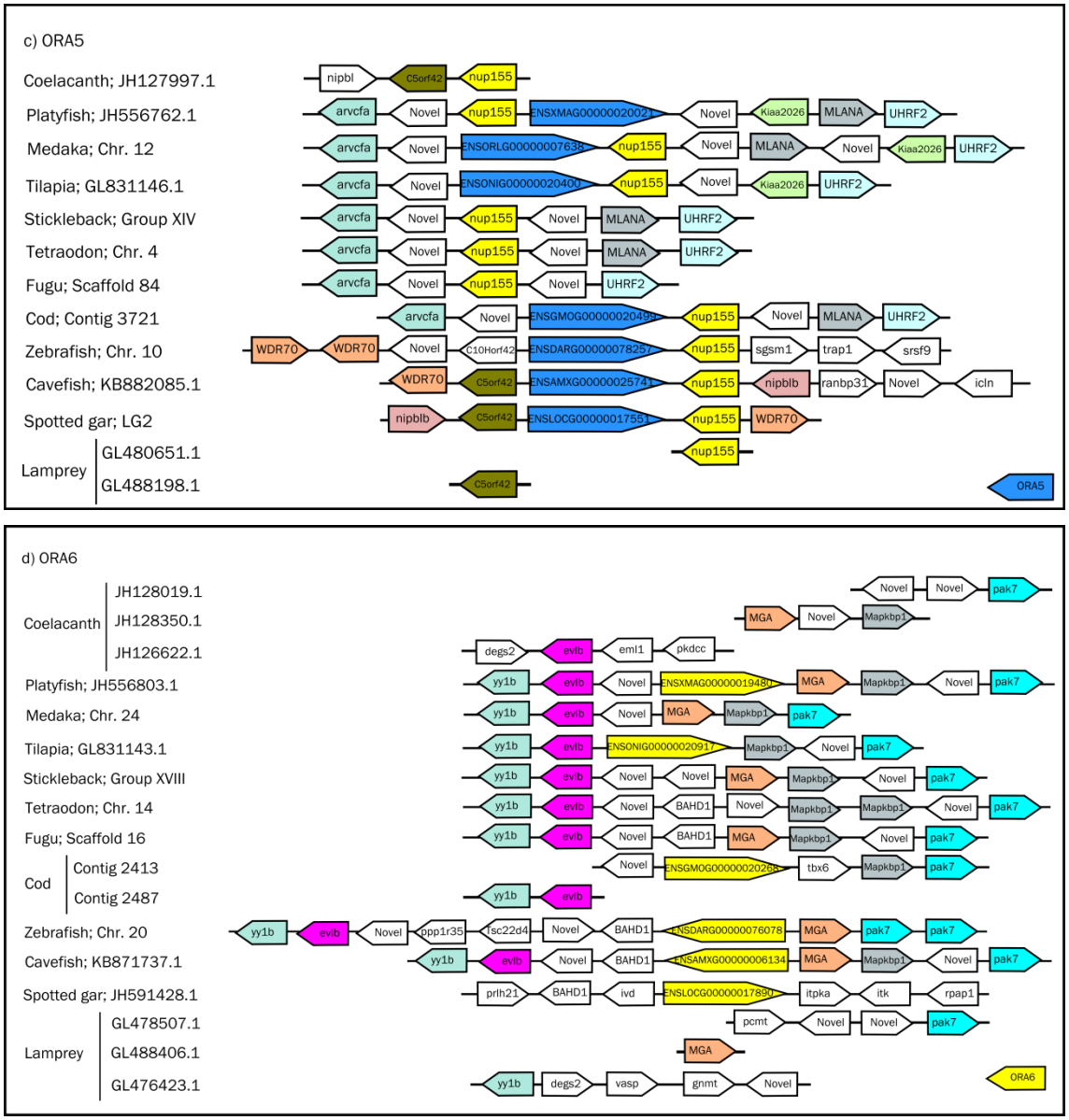



Figure 10 - Location of Ora 1 and Ora 2 (a), Ora 3 and Ora 4 (b), Ora 5 (c) and Ora 6(d) genes and respectively flanking genes.

OlfC genes were also related with pheromone detection in fishes and if in one hand, similar to Ora, they have low percentage of pseudogenization; in the other side they have a high variation in repertoire size among species (Johnstone, Ciborowski et al. 2009). OlfC genes are usually encoded by six exons separated by introns with fully conserved structure (Hashiguchi and Nishida 2005, Alioto and Ngai 2006). It could have some variations in exon length between and within subfamilies but the phases of intron/exon boundaries within codons are strictly conserved across all members of the family (Alioto and Ngai 2006).

It were reported that OlfC genes are usually organized in big well-defined genomic clusters where genes in the same subfamily tend to be located close to one another in the same transcriptional orientation with similar arrangement and position (Hashiguchi and Nishida 2006). We explored this point, approaching available information deposited in Ensembl database for medaka, green spotted pufferfish (tetraodon), platyfish, stickleback, cavefish and spotted gar. We concluded that OlfC genes form big clusters usually flanking by neprilysin and η-type phospholipase C (PLC-η) (Figure 11), as reported by other authors for Lake Victoria cichlid, Atlantic salmon and zebrafish (Johnstone, Ciborowski et al. 2009, Nikaido, Suzuki et al. 2013). However, zebrafish clusters only maintain PLC-η gene as flanking landmark. These features suggest a common ancestor following by tandem duplication as the primary mechanism for expansion of this gene family (Hashiguchi and Nishida 2006).

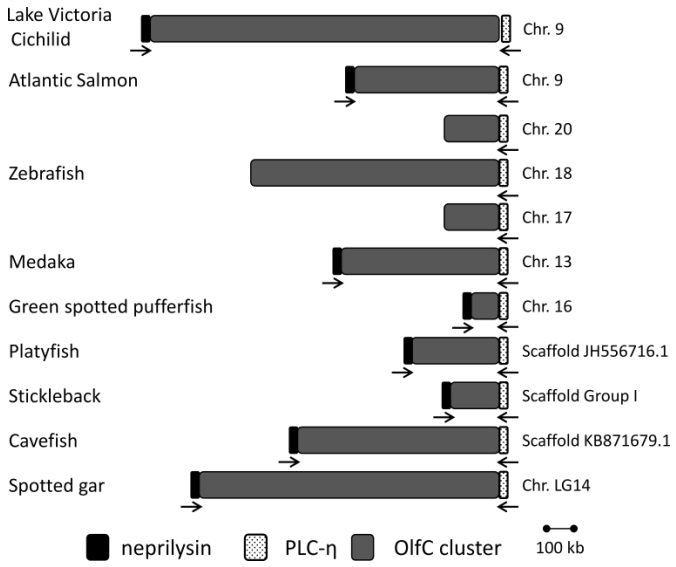



Figure 11 - The chromosomal location of the major OlfC gene clusters and conserved synteny of the flanking genes. PLC-η gene is present in the end of all clusters whereas neprilysin gene is not present in zebrafish and in Atlantic salmon clusters. Information for medaka, green spotted pufferfish, platyfish, stickleback, cavefish and spotted gar were retrieved from Ensembl database whereas Lake Victoria cichlid, Atlantic salmon and zebrafish information were based on previous studies (Johnstone, Ciborowski et al. 2009, Nikaido, Suzuki et al. 2013).

Previous work from other authors reported that OlfC genes could be divided into 17 subfamilies (Johnstone, Ciborowski et al. 2009). Using complete fish OlfC sequences available in Ensembl database, we reconstructed the phylogenetic tree of this gene family. As the majority of OlfC genes in Ensembl database were not classified, we used the complete sequences of *Danio rerio* dataset reported by Hashiguchi and Nishida in 2006, in order to classify the uncharacterized fish OlfC sequences. CaSR genes were used as out-group.

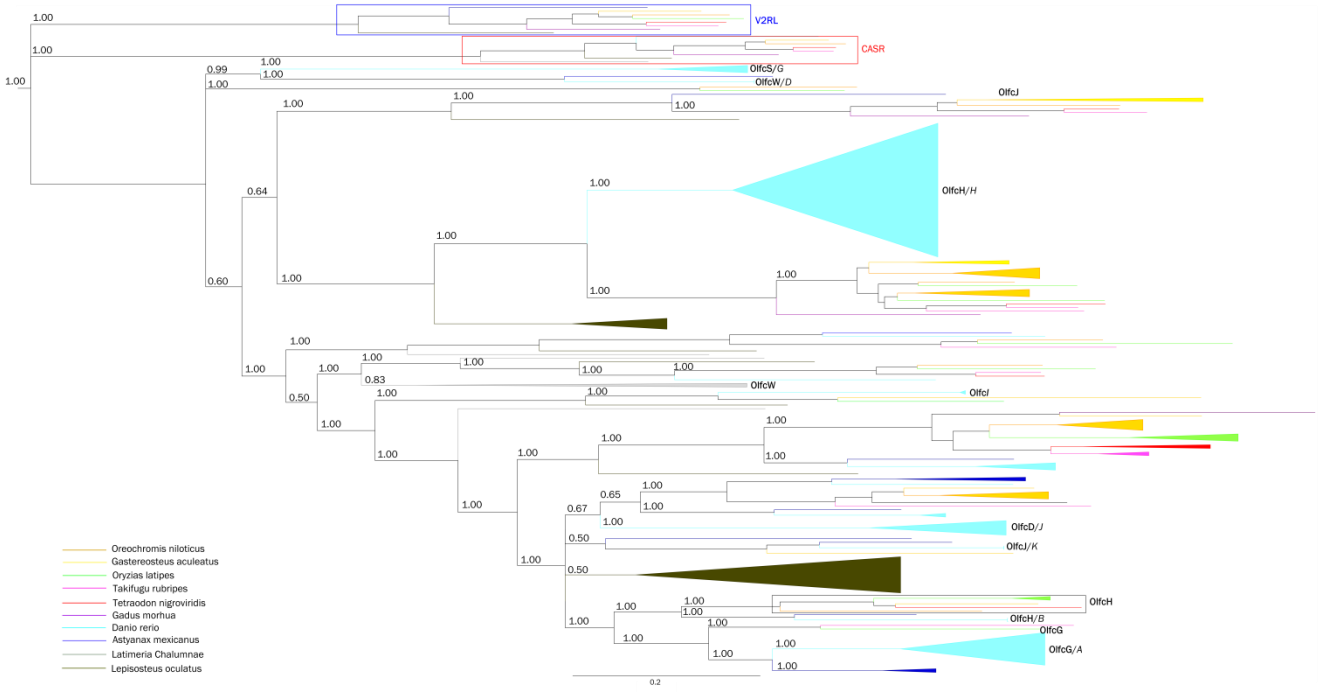



Figure 12 – Bayesian tree of OlfC genes. Bayesian tree were reconstructed using MrBayes software version 3.2.2 with OlfC genes available in Ensembl database with1000000 generations and following the GTR+I+G model. The first twenty fiver percentage of results was discarded for the calculation of the posterior Bayesian probability.

Analysing our Bayesian tree we concluded that some annotated “V2R-like” genes in fishes stay close to the CaSR cluster, in outside position. For all the other OlfC genes we detected some species-specific duplications in a complex tree arrangement that do not allow identification of 17 proposed subfamilies (Johnstone, Ciborowski et al. 2009). In addition to that the OlfC *Danio rerio* nomenclature suggested by Hashiguchi and Nishida in 2006 is completely divergent of those annotated in Ensembl database (only OlfCH genes share the same family name). More studies in further are needed to characterized OlfC genes and demystify its complex phylogeny.

In addition to pheromone sensing, it was reported that OlfC family might also act as amino-acid sensing-receptors (Pfister and Rodriguez 2005). In goldfish and zebrafish it was reported two orthologous receptors that are activated by amino acids (Speca, Lin et al. 1999, Luu,Acher et al. 2004) and, at the same time, it were reported that all

zebrafish OlfC genes have the eight conserved amino acids signature motif characteristic of amino acid-sensing ligand-binding receptors, in which five of them interact directly with amino acids (Acher and Bertrand 2005, Alioto and Ngai 2006). All of our analyzed OlfC genes present the eight conserved amino acids (Figure 13). In amino acid binding receptors the binding pocket can be divided into proximal pocket (with residues that interact with the glycine moiety of the amino acids) and distal pocket (with residues that interact with the group R of side chain) (Pin, Kniazeff et al. 2004). In zebrafish and fugu OlfC receptors it was reported a high degree of conservation of proximal binding pocket residues but low degree of conservation in distal pocket, which suggest that OlfC genes are able to detect and discriminate a diverse spectrum of amino acids (Alioto and Ngai 2006). In addition to that, the high OlfC repertoire of cichlids supports the keen ability that this species present in discriminate a high variety of amino acids, related with their observed extraordinary diversification of feeding behaviors (Nikaido, Suzuki et al. 2013). However, zebrafish OlfC genes appear to be under negative selection (Alioto and Ngai 2006) but no more information is actually available for OlfC genes in other fish species.

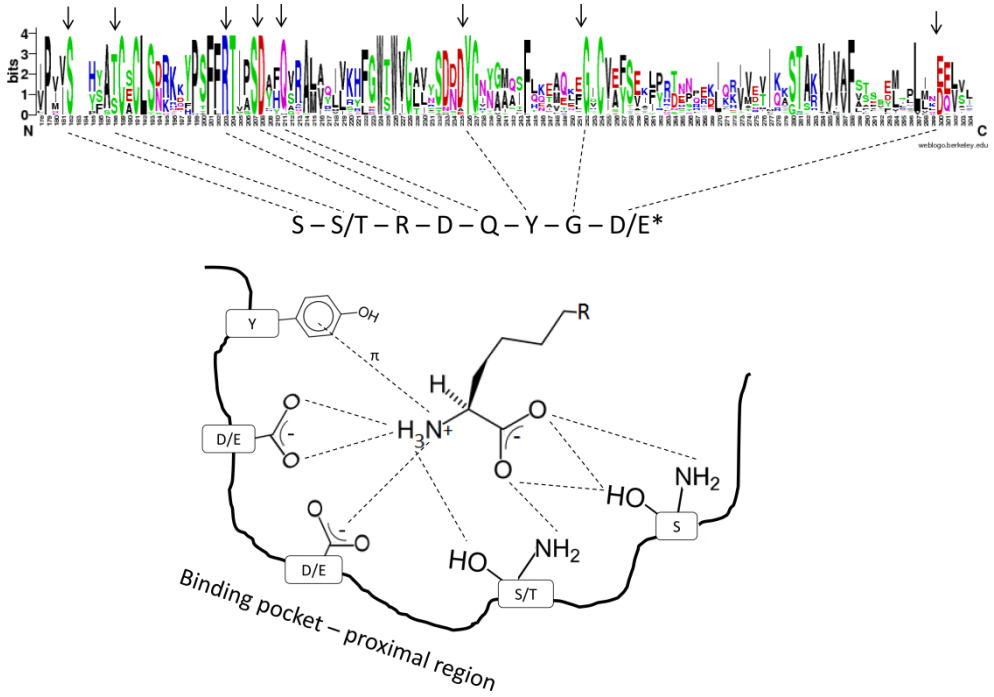



Figure 13 – Conserved residues inside proximal region of the OlfC binding pocket.

# Conclusions and future perspectives

Detection and identification of chemical cues are essential for species survival since sensory perception is crucial for both inter and intra-species relationships. The detection of pheromones has high impact in intraspecific communication and is essential for mating. The discovery of vomeronasal receptors as main interveners in pheromonal communication was the first step to understand the pheromones perception “world”.

Analysing the distribution and variation of vomeronasal receptors, type 1 and type 2, we can detect a non-uniform and non-widespread distribution across vertebrates as previously recognized. Vomeronasal receptors are tetrapods specific and have appeared firstly in amphibians. Furthermore, these receptor repertoires have a dynamic evolution but do not appear be connected with the transition of species from water to land environment or domestication processes.

Considering our genomic searches, we conclude that presence of vomeronasal communication could be closely connected with the presence of the vomeronasal organ. In species that it was reported loss of the vomeronasal organ, 1) vomeronasal receptors were not identified and pheromonal communication was not detected, or 2) pheromones are detected by other systems like in the case of fishes. In addition, TRPC2 gene appears to be necessary for pathways involved in pheromonal detection by VRs, ORAs and OlfC receptors.

However, even in tetrapods there is a gap in vomeronasal communication of some groups of species like reptiles. Despite it is known that chemical sense in squamates reptiles is extremely important for foraging, (Saviola, Chiszar et al. 2013) avoidance of predators and social relationship (Cooper 1994, Martin and Lopez 2000, Cooper and Pèrez-Mellado 2002, Shine and Mason 2012), the number of studies on the components of the vomeronasal system in reptiles, is still very reduced. Moreover, the reduce information available on reptilian genomes is a barrier to achieve further information in vomeronasal communication in squamates reptiles.

The approach of vomeronasal receptors requires the study of complex gene structures (e.g. the six characteristic exons in V2Rs genes), with multiple gene copies in some species (e.g. extensive reptilian V2Rs or rodents V1Rs repertoires). Moreover, the absence of conserved synteny and orthology between VRs genes in vertebrates and their structural similarity with other chemosensory receptors have make difficult their identification and the reconstruction of the vomeronasal communication history across vertebrates. Nevertheless, the development of new bioinformatics tools and the future

increase number of reptilian and amphibian available genomes should provide new achievements in the pheromonal communication field.

Understand which receptors are involved in the detection of pheromones will be crucial to identify their ligands, which are actually unknown for the majority of vertebrates. Solving these remaining points, and being pheromonal communication so important in attraction and mating in several vertebrates, currently problems in the behaviour and reproduction of some wild and captive species could be easily perceived.

# Chapter B – Detoxification

## Introduction

Detoxification and elimination of drugs and endogenous metabolites are crucial for metabolic homeostasis, being xenobiotics metabolized by a large number of xenobiotics metabolizing enzymes which fall into three broad categories: phase I, II and III. Age and gender, diet and lifestyle, environment, diseases and genetic polymorphisms are some of the main factors that influence the detoxification activity.

Phase I enzymes are mainly monooxygenases, able to converts hydrophobic xenobiotics into more hydrophilic molecules, such as cytochrome P450 family complex, alcohol dehydrogenase and aldehyde dehydrogenases. In phase II occurs the conversion of Phase I products into amphiphilic anionic conjugates that are water soluble by addition of glucuronic acid, glutathione, sulphate or acetyl molecules (Rowland 2013). In this phase the main interveners are glutathione transferases, UDP-glucuronyl transferases, carboxylesterases and sulfotransferases, among others. In Phase III conjugated xenobiotics are exported out of the liver by transporters such as ATP binding cassette subfamily members, organic anionic and cation transporters and solute carriers (Lee 2011).

**Table 1 – Function and examples of enzymes involved in detoxification phase I, II and III.**

| | **Phase I enzymes** | **Phase II enzymes** | **Phase III enzymes** |
|---|---|---|---|
| **Function** | Conversion of hydrophobic molecules into more hydrophilic compounds. | Conversion of phase I products into amphiphilic anionic conjugates. | Exportation of conjugated compounds out of the liver. |
| **Examples** | *Cytochrome P450 family<br>*Alcohol dehydrogenase<br>*Aldehyde dehydrogenase | *Glutathione Transferases<br>*UDP-glucuronyl transferases<br>*Sulfotransferases<br>*Epoxide hydrolase<br>*N-acetyl transferases<br>*Carboxylesterases | *ATP binding cassette family<br>*Several transmembranar transporter |

The Cytochrome P-450 (CYP) are a superfamily of genes which compose the detoxification Phase I core system, being thus of high importance for species metabolism. These genes are expressed within the endoplasmic reticulum of the liver – the main intervener in the detoxification Phase I. CYPs are important not only in xenobiotic detoxification, but also in the synthesis and degradation of endogenous compounds, such as hormones, prostaglandins and vitamins. It was reported that usually the CYPs involved in endogenous substrate metabolism are more stable than those involved in the detoxification of exogenous compounds, which often have a high frequency of non-synonymous substitutions at the substrate recognition site (Gotoh 2012).

Its nomenclature system is based on a hierarchical clustering of CYPs into i) families if the sequences have more than 40% of amino acid identity and ii) subfamilies if they present more than 55% of amino acid identity (Gotoh 2012). Actually, there is no consistency regarding the exact number of CYP elements (functional genes or pseudogenes) existing across different species, which could be related with the high frequency of gene-gain and gene-loss events after lineage divergence (Feyereisen 2011, Uno 2011). This fact makes difficult to determine orthologous relationships of CYP450 between species (Uno 2011). Furthermore, CYP450s present several polymorphisms such as gene deletions, gene duplications, SNP or frameshift mutations. These polymorphisms are responsible for variations in amino acid structure and changes in substrate specificity, which result in different drug metabolization rates between elements of the same family (Wang 2010, Feyereisen 2011).

Active CYP genes codify enzymes usually with over 400 residues length, often with some highly conserved regions, such as proline-rich cluster close to the amino (N)-terminus and a heme-group that link a cysteine sulfur atom to form an iron-sulfur (Fe-S) bond (Wang 2010). The hydroxylation of substrate (RH) involves incorporation of one oxygen atom from $O_2$ molecule to originate a ROH product. The other oxygen atom is reduced to $H_2O$ by receiving hydrogen atoms from nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 14). Inside this pathway the Fe-S bond is crucial for the electron transport from NADPH donor to the substrate, nevertheless the intermediate step is not fully understood (Nelson and Cox, Wang 2010). In general, the products of CYPs are more soluble and hydrophilic, being able to be further processed by Phase II enzymes. However, sometimes the hydroxylation process could convert the substrates into products that are more toxic than the original compounds, subverting the detoxification system (Nelson and Cox 2008).

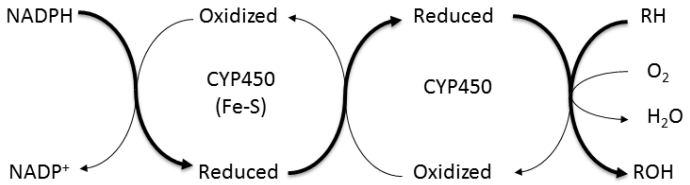



**Figure 14 – Hydroxylation pathway by CYP450 enzymes using NADPH as electron donor.**

The CYP activity and its expression could be related with the biologic clock, since rodents CYP 2 family, subfamily A, member/polypeptide 1 (CYP2A1) – which encodes testosterone 7-α-hydrolases – shows circadian alterations and CYP 2 family, subfamily E, member/polypeptide 1 (CYP2E1) – involved in metabolization of small and hydrophobic compounds – have a significant 24h hepatic rhythm in messenger ribonucleic acid (mRNA) level and protein activity (Froy 2009). Moreover, other detoxification enzymes dependent on CYP also show the same rhythmic effect suggesting a possible circadian detoxification of a large number of drugs. Other detoxification enzymes dependent of CYP450 also show the same rhythmic effect which suggest a possible circadian detoxification of large number of drugs (Froy 2009).

The detoxification Phase II involve different kinds of enzymatic systems but both have the main objective to transform the Phase I products in more hydrophilic compounds by addition of key molecules. UDP-glucuronyl transferases (UGTs), γ-glutamyltranspeptidases (γ-GTPs), glutathione s-transferases (GSTs) and carboxylesterases are some examples of enzymes involved in this step.

UGTs catalyze reactions known as glucuronidation, which consist in the transfer of UDP-glucuronic acid to a lipophilic substrates that could be endogenous compounds (bilirubin, bile acids, fatty acids, steroid hormones, thyroid hormone) or exogenous substances (analgesics, non-steroid anti-inflammatory agents, antipsychotics, antivirals, benzodiazepines). Glucuronidation is extended to all mammalian species, however, with significant variation in enzymes substrates selectivity and rates of reaction (Rowland 2013). Despite it were reported only four UGT families, large number of enzymes are reported due to alternative splicing and as CYPs, in UGTs different polymorphisms are related with distinct abilities in drugs metabolizing between and within species (Rowland 2013).

γ-GTS hydrolysis γ-glutamyl bonds of glutathione and glutamine, generating a free γ-glutamyl group that are transferred to amino acids or short peptides. γ-GTS are conserved enzymes, highly expressed in kidney, liver and heart, that have important roles in glutathione metabolism and in detoxification and inflammatory processes (Castellano 2012).

Glutathione (GSH) is also a key residue in GST activity. GST enzymes are responsible for conjugation of glutathione with Phase I products that have electrophilic center and are involved in protection against chemical toxicity (both endogenous and exogenous metabolites) and oxidative stress (Hayes 1995, Oakley 2011, Raza 2011). GSTs are involved in insecticide resistance by the 1) neutralization of organophosphates and DDT, and by 2) neutralizing the oxidative stress induced by insecticides (Perera, Hemingway et al. 2008, Che-Mendoza, Penilla et al. 2009, Wu, Dou et al. 2009).

If in one hand GST levels can be increased by expose to foreign compounds, in other hand GST can by it-self modulate the induction of other detoxification enzymes such as quinine reductase, aflotoxin-B1 aldehyde reductase, UDP-glucuronosyl transferase, γ-glutamyl transferase and γ-glutamylcysteine synthetase, among others (Hayes 1995). GST's are widely distributed in nature and can be classified as cytosolic, membrane bound, microssomal or mitochondrial GTS (Raza 2011).

Carboxylesterases are another group of enzymes involved in detoxification phase II and are involved in hydrolysis of lipophilic xenobiotic (Sogorb 2002), such as the phosphotriesters molecules presents in insecticides (Bigley 2013).

Detoxification Phase I and II are closely connected and an illustrative example is the metabolization of Aflatoxin B1. Aflatoxin B1 is a metabolite from some *Aspergillus* species and is frequently a contaminant present in cereals and tree nuts that are the feeding base of several species (Wu, Jezkova et al. 2009).

After being ingested, Aflatoxin B1 is activated by CYP450 complex into the AFBO product. However, AFBO is more toxic than its precursor, with high degree of toxicity that induces mutations, cancer and death (Rawal 2010). The only way to eliminate AFBO is by GST metabolization that conjugate AFBO with GSH in order to form an adduct that is easily excreted (Rawal 2010) and wild turkeys, that have AFBO trapping functional GST alpha, are extremely resistant to this toxic intermediate (Kim, Bunderson et al. 2013) (Figure 15).

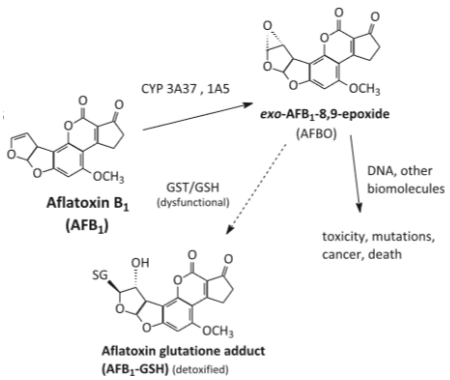



**Figure 15 – Metabolic pathway of Aflatoxin B1 epoxidation by CYP3A37 and CYP1A5 and posterior conjugation with GSH in order to be detoxified (Rawal 2010).**

After conversion of hydrophobic substances into more soluble ones by Phase I and Phase II detoxification enzymes, the compounds could be easily excreted. In these step ATP-binding proteins as other transmembranar proteins are crucial to pump xenobiotics out of a cell in order to decrease their concentration (Lee 2011).

Despite xenobiotics that are neutralized by detoxification enzymes, the organisms also have to deal with endogenous reactive species. Reactive oxygen species (ROS) are naturally produced in metabolic pathways and directly oxidize several physiological molecules such as catecholamines, polyphenols and leucoflavins, which also implicate strategies to neutralize and eliminate ROS (Montgomery, Hulbert et al. 2011). Three main lines of defence are known with involvement of cytochrome oxidases in reduction of dioxygen (first line), action of other enzymes that remove the intermediates generates after dioxygen reduction (second line) or use of antioxidants to prevent the initiation and/or propagation of chain reactions. Super oxide dismutase (SOD) is the main enzyme involved in dismutation of $O^{2-}$ into $H_2O_2$ (Fridovich 1989). Posteriorly $H_2O_2$ is dismuted into $H_2O$ and $O_2$ by catalase or peroxidases (Figure 16).

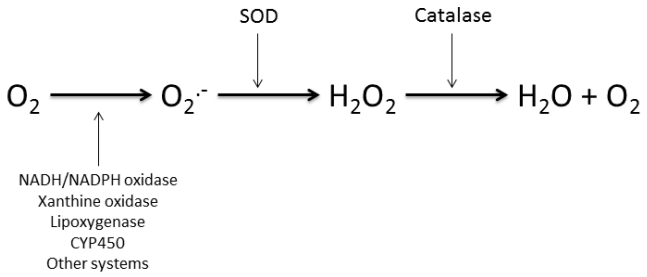



**Figure 16 – Involvement of SOD and Catalase in neutralization of ROS. Figure adapted from Kyaw, Yoshizumi et al. 2004.**

SOD can be classified into three main families: MnSOD (present in prokaryotes and in mitochondrial matrix), FeSOD (present in prokaryotes and few families of plants) and Cu,ZnSOD (present in eukaryotic cells and chloroplasts). Despite of MnSOD and FeSOD are structurally and functionally more similar, Cu,ZnSOD have different characteristics, which suggest that these enzymes should had an independent evolutionary history (Frifovich 1989). The ubiquitous cytosolic Cu,ZnSOD function in homodimeric organization with each subunit binding one zinc and one copper atom (Pasinelli 2006) in order to alternatively reduce and re-oxidate the Cu(II), interacting with $O_{2}^{\cdot -}$ and dismute it into $H_2O_2$ (Fridovich 1989). $H_2O_2$ is in the next step degraded by catalase enzymes. It is known two classes of catalases (“monofunctional” catalase and catalase-peroxidase) that despite differences at sequence and structure level, maintain similar activity (Zamocky, Furtmuller et al. 2008). Monofunctional catalase enzymes are most widespread in nature but share with catalase-peroxidase two-stage in $H_2O_2$

degradation mechanism, being the first one the oxidation of heme group into oxyferryl species by one hydrogen peroxide molecule and the second step the utilization of other hydrogen peroxide molecule as a reducer to regenerate the resting-state of catalase and originate water and oxygen (Chelikani 2004). The levels of $H_2O_2$ can also be regulated by metazoan peroxiredoxin enzymes (Wood 2003, Poole 2011, Brinkmann 2013).

As it was referred, animal species have sophisticated enzymatic systems able to metabolize dangerous molecules. However, they could be in contact with metals that depending on their bioavailability and concentration are potentially toxics (Lin 2012). The strategies developed by animal species are based on neutralization systems that trap the metals, blocking their nefarious effects. Metallothioneins (MTs) are elements of these trap systems since they encoded low molecular weight cysteine-rich proteins (Scudiero, Temussi et al. 2005, Braune and Scheuhammer 2008) able to bind not only essential metals like zinc or copper, but also toxic metals like cadmium, mercury, platinum and silver, in a total of 7 to 12 metal atoms per molecule (Pal 2010, Takahashi 2012). Due to this dual ability to bind both essential and toxic metals, MTs may play an important role in hematopoietic cell proliferation and differentiation and also have functions in drug resistance, protective against oxidative stress and metal toxicity (Takahashi 2012). MTs can also be involved in protection against reactive gases and radiations (Namdarghanbari 2011). MTs superfamily is subdivided into 16 families, with only one family present in vertebrates and all the other fifteen distributed across invertebrates, fungi kingdom, prokaryotes and plants, and their expression is induced by contact with metals (Höckner, Dallinger et al. 2011).The study of MTs is important due to the environmental pollution by metals and the necessity of understanding molecular mechanisms involved in their toxic effects. In addition to that, induction of MTs and their capacity to bind metals has been proposed as potent biomarkers of metal stress (Nam, Kim et al. 2007). However, little is known about MTs origin and differentiation patterns (Trinchella, Riggio et al. 2008) and MTs evolution is still an unsolved aspect (Trinchella, Esposito et al. 2012).

In this chapter, we studied two concrete examples of enzymes involved in detoxification Phase I and Phase II, the family 2 of CYP450 complex (CYP2) and cytosolic GST (cGST) family, respectively, in avian species. Birds have a high metabolic rate (Munshi-South and Wilkinson 2010), high lifetime energy expenditure (Mushi-South and Wilkinson 2010) with constant high blood glucose levels (Holmes and Ottinger 2003), seasonal high blood lipid levels (Holmes and Ottinger 2003) and high body temperature (Montegomery, Hulbert et al. 2011). All of these characteristics should lead to low lifespan but birds have almost twice higher lifespan that similar size species, namely mammals (Montgomery, Hulbert et al. 2011). Their high lifespan could be due to

defense mechanism to combat oxidation and glycoxidation damage (Holmes and Ottinger 2003) in order to confer oxidative stress resistance (Munshi-South and Wilkinson 2010). In addition to that it was reported that birds have a reduced level of ROS (Buttemer, Abele et al. 2010, Montgomery, Hulbert et al. 2011), but the mechanism that prevent their formation or its extremely efficient elimination is still unknown (Montgomery, Hulbert et al. 2012). Moreover, it was reported in insects an expansion of genes related with antioxidant defense, such as GSTs, that have important role in detoxification of reactive oxygen species generated during fight (Wang, Fanf et al. 2014).

By these reasons, the main goal of this study was to understand the rapid evolution of avian CYP2 and cGST genes and relate the results with their function in the detoxification process.

## Material and methods

### Avian CYP450 family 2 sequences and phylogenetic analysis

BLAST searches of the known vertebrate specific CYP450 family 2 (CYP2) subfamily members, available in NCBI database, were performed against 45 fully sequenced genomes from the Avian Genome Consortium. The dataset of nucleotide sequences obtained was organized in several CYP2C subfamilies. All the subfamilies were aligned by codons - in order to keep the correct reading frame - using the MUSCLE programme, available in SeaView software version 5.3.3 (Gouy, Guindon et al. 2010). Saturation analyses i) plot of the transitions and transversions versus divergence and ii) measure substitution saturation through the Test by Xia et al. (2013), were conducted using DAMBE software version 5.3.3 (Xia 2013). Recombination sites were identified using the GARD programme available in the Datamonkey (Kosakovsky Pond, Posada et al. 2006) and RDP3 software (Martin, Lemey et al. 2010) webserver. The sequences with recombination events were discarded for further analyses. A final alignment with all the CYP450 sequences was created, using the same methodology previously described, and then a Maximum Likelihood (ML) phylogenetic tree was reconstructed in the PhyML software version3 (Guindon, Dufayard et al. 2010). For this phylogenetic analysis we considered 1000 bootstrap replicates and the previously determined best-fitted model of nucleotide substitution, GTR+I+G, following the Akaike (AIC) model detected by jModeltest2.1.1 (Guindon 2011, Darriba D., Taboada G.L. et al. 2012).

Avian GST sequences and phylogenetic analysis

GST alpha, zeta, theta, pi and omega from five available bird genomes were downloaded from the Ensembl database. Since the GST mu class was not identified in the avian genomes, further searches were conducted in the NCBI database. All sequences ID are present in annex. Because the high sequence divergence of cGSTs, they were aligned based on structure. A maximum likelihood (ML) phylogenetic tree was constructed in PhyML software version 3 (Guindon, Dufayard et al. 2010), using TVM+I+G model and 1000 bootstraps replicates. A Bayesian tree was constructed in MrBayes software version 3.2.2 (Ronquist and Huelsenbeck 2003) using GTR+I+G model with 1 cold and four incrementally heated chains, T=0.2, for 2000000 generations and discarding the first 25% of the results in the posterior Bayesian probability.

Three-dimensional GST structures prediction

The structures of chicken GST alpha and mu were retrieved from the PDB database (1VF1 and 1GSU, respectively). Human three dimensional sequences of GST zeta, omega, sigma and theta (ID 1FW1, 1EEM, 2VCZ and 2C3N, respectively) were used as query for predicting the structure of the chicken GST zeta (NP_001264391.1), GST omega (E1BX85), GST sigma (O73888) and theta (E1BUB6), respectively, using the Swiss model workspace. The QMEAN4 score, a linear combination of 4 statistical potential terms that estimate model reliability (value between 0-1), was higher than 0.75 for all the predicted GST structures. Visualization representations were created with VMD visualization software (Humphrey, Dalke et al. 1996).

## Results and discussion

Avian CYP2 family

CYP450 superfamily is the main core of Phase I detoxification. In fact elements from families 1, 2 and 3 are responsible for 70-80% of the detoxification metabolism that occur in Phase I. Usually they present less affinity for their substrates, low evolutionary conservation and important genetic polymorphisms. The avian CYP2 elements were studied with the main objectives of i) understanding gene duplication patterns, ii) clarify the phylogeny of CYP2, iii) identify sites under positive selection and iv) interpret the results considering the various ecological features of the studied avian species. After the

blast searches on the avian genomes we retrieved a dataset of genes that was properly annotated and represented in Figure 17.

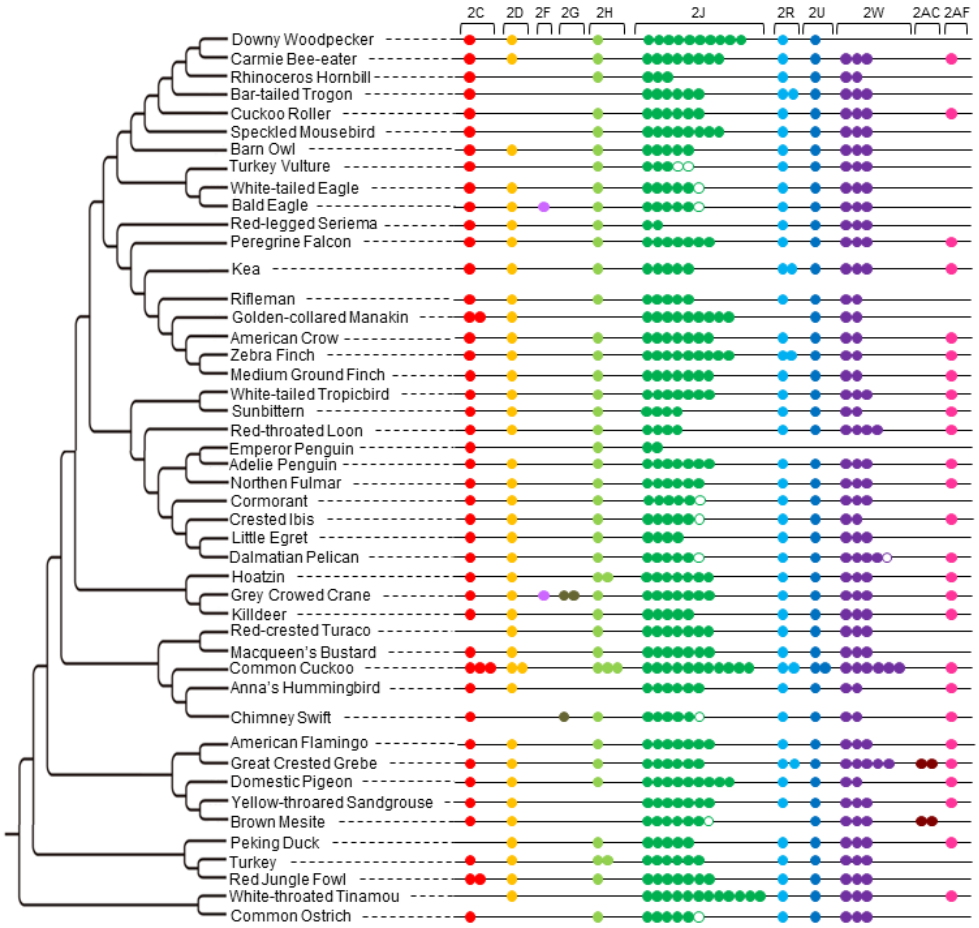



**Figure 17 – Number of elements from each CYP2 subfamily detected in the avian species. Full and open circles represent genes and pseudogenes, respectively.**

We found avian genes corresponding to the following CYP2 subfamilies: C, D, F, G, H, J, R, U, W, AC and AF.

All analyzed birds present a single element of CYP2C subfamily (Watanabe, Kawai et al. 2013), with the exception of chicken, golden-collared manakin and common cuckoo. It was suggested that a single CYP2C ancestor gene duplicated independently in avian and human lineages since there are not a clearly orthology between CYP2C genes in both species (Watanabe, Kawai et al. 2013). It was reported that CYP2C genes should have an important role in chicken xenobiotic metabolism (Watanabe, Kawai et al. 2013) because they are activated by the chicken xenobiotic receptor (CXR) (Baader, Gnerre et al. 2002). Furthermore, CYP2C45 of chicken LHM cells (cell line of chicken liver) could be induced by rifampicin, β-naphtho-flavone, dexamethasome, cloritanazole and scoparone (Baader, Gnerre et al. 2002, Cai, Jiang et al. 2012) and in cormorant, perfluorochemicals were identified as strong CYP2C activators (Kubota, Stegeman et al. 2011). However, the CYP2C subfamily should also be involved in endogenous

metabolism of polyunsaturated fatty acids (PUFAs) because it was reported higher levels of CYP2C in lean chickens than in fat ones, which suggest the involvement of CYP2C enzymes in the down-regulation of the lipid metabolism in order to control the availability of PUFA and reduce of quantity of fat tissue (Carré, Bourneuf et al. 2002).

It was reported a single avian copy belonging to the CYP2D subfamily (Watanabe, Kawai et al. 2013) and the majority of our bird species analyzed are concordant with this pattern with exception of the common cuckoo and chicken that in both cases have two copies. In some mammals CYP2D is extendedly duplicated, such as in rodents with 7 copies, primates with 2-3 copies, rabbit with 5 copies and horse with 6 copies, which can be related with feeding habits and the affinity of CYP2D6 for metabolizing plant toxins like alkaloids (Yasukochi and Satta 2011). The endogenous activity of CYP2D genes was proved by the ability of chicken CYP2D49, which is similar to human CYP2D6, to metabolize bufuralol, a β-adrenoreceptor antagonist whose effects are blocking endogenous catecholamines, inducing the smooth muscle relaxation and lipolysis (Cai, Jiang at al. 2012). According to our knowledge, no studies are available regarding the metabolic rates of avian CYP2D6. However, human CYP2D6 is responsible for metabolizing 25% of therapeutic drugs and also has high affinity for alkaloids (Yasukochi and Satta 2011).

In chicken it was reported the presence of two elements of the CYP2H subfamily (Davidson, Dogra et al. 2001), which share 92% of identity, suggesting a very recent gene duplication that probably was not shared by other birds (species-specific) (Kubota, Stegeman et al. 2011). The majority of the analyzed birds, including chicken, only possess one gene but in common cuckoo, hoatzin and turkey it was found extra copies that are more likely to be related with multiple duplication events.

Single elements were also reported for CYP2R and CYP2U subfamilies (Watanabe, Kawai et al. 2013). The majority of our bird species analyzed present one copy, with the exception of a few species (common cuckoo in CYP2R and CYP2U, kea, great crested grebe and zebra finch in CYP2R and bar-tailed trogon in CYP2U). The duplication of zebra finch CYP2R was previously reported by other authors (Kubota, Steheman et al. 2011). A conserved synteny is shared between avian and human CYP2R and CYP2U genes, which support a high degree of conservation despite the different lineages evolution (Watanabe, Kawai et al. 2013). This could be related with the fact of both subfamilies possess an essential and conserved role in the metabolism of vitamin D and arachidonic acid (Watanabe,Kawai et al. 2013).

Previously studies (Kubota, Stegeman et al. 2011) reported one CYP2AF gene in cormorant and zebra finch. Nevertheless, in our analysis this gene was not detected in the referred species, being present one single copy in other analyzed avian species.

In addition to that, CYP2AF was being identified as avian-specific (Kirischian, McArthur et al. 2011).

In the referred subfamilies, the majority of the species only possess one copy of each gene, being the gene duplication isolated events. However, common cuckoo is an exception with several duplications in all the considered subfamilies, which suggest that this species could have suffered different patterns of evolution.

In other CYP2 subfamilies few elements were identified. However, they are not present in all the analyzed species. The CYP2F subfamily was previously suggested to be a mammalian-specific subfamily (Kirischian, McArthur et al. 2011) but our analysis identified a single copy in grey-crowed crane and bald eagle, similar to the CYP2F subfamily. Likewise, CYP2G subfamily was also proposed to be mammalian-specific (Kirischian, McArthur et al. 2011) but recently elements of this subfamily were detected in the anole lizard, with at least seven copies (Watanabe, Kawai et al. 2013, Kubota, Stegeman et al. 2011). In our dataset we identified a single copy in chimney swift and two copies in the grey-crowed crane. We only detected CYP2AC elements in two species but in both we found two copies that contrast with the single copy found in the human repertoire (Watanabe, Kawau et al. 2013).

On one hand, the majority of CYP2 subfamilies in birds have one or few duplicated elements, while additional subfamilies such as CYP2J and CYP2W present high number of elements.

The CYP2J subfamily in our analyzed avian species possesses 3 to 12 genes. Previously studies reported 6 genes in chicken, 4 in zebrafinch and 3 in turkey (Watanabe, Kawai et al. 2013). Our analysis identified 7, 9 and 6 genes, respectively. These avian results contrast with the non-duplicated scenario of the human repertoire (Watanabe, Kawai et al. 2013) but duplications are reported in other mammals like rats with 5 genes and mice with 8 copies (Kubota, Stegeman et al. 2011). It was also detected similar duplication events in Bactrian camels that can be explained by the fact of CYP2J helps in the conversion of arachidonic acid into (19S)-Hydroxyeicosatetraenoic acid (19(S)-HETE) that is a potential vasodilator of renal pre-glomerular vessels, stimulating water reabsorption (Consortium 2012). Other study reported that CYP2J has epoxigenase activity and can convert arachidonic acid in epoxycosatrienoic acids (EETS), which are an anti-hypertensives with vasodilatory properties (Yu, Huse et al. 2000). So it was hypothesized that the number of CYP2J could be proportional to the capability of water absorption and survival in dry conditions (Consortium 2012). In fact, the water retention is crucial in birds, mainly in migratory species.

In CYP2W we also found 2 or more copies in all the analyzed species. It was reported that CYP2W1, in chicken and mammals, are orthologous and syntenic to fish

CYP2K elements (Nelson 2011), and it was suggested that both subfamilies should arrived from whole genome duplication events that also affected the CYP2D precursor (Nelson, Goldstone et al. 2013).

Previously reported, the presence of avian elements inside CYP2A and CYP2AB subfamilies, such as chicken and quail CYP2A6 genes, involved in the bioactivation of AFB1 (Diaz, Murcia et al. 2010), or the 5 and 4 copies present in chicken and zebrafinch CYP2AB subfamily, respectively (Kubota, Stegman et al. 2011). However, our analyzes did not revealed none of these genes in our avian dataset.

The absence of some subfamilies could be related with the high fragmentation and low quality of some analyzed genomes, which maintain the door open for further investigations in order to achieve a more complete repertoire of some CYP subfamilies.

We did not find genes of the subfamilies B, E, K, N, P, Q, S, T, V, X, Y, AA, AD, AE and AG, which is in accordance with earlier studies that excluded these subfamilies from birds (Kirischian, McArthur et al. 2011, Kubota, Stegeman et al. 2011, Watanabe, Kawai et al. 2013).

All the detected CYP2 elements were grouped by subfamilies in order to facilitate the alignment process. As in some families we found duplicated elements, we evaluated the presence of saturation (Xia 2013) and recombination events (Kosakovsky Pond, Posada et al. 2006, Martin, Lemey et al. 2010) with the objective to discard sequences with evidence of saturation and/or recombination. This step is crucial since these events could affect the accuracy of the phylogenetic analyses and positive selection analyses, by originating false positive results. The resultant sequences were used to reconstruct a Maximum Likelihood phylogenetic tree, based on GTR+I+G model previously detected by jModeltest (Figure 18).

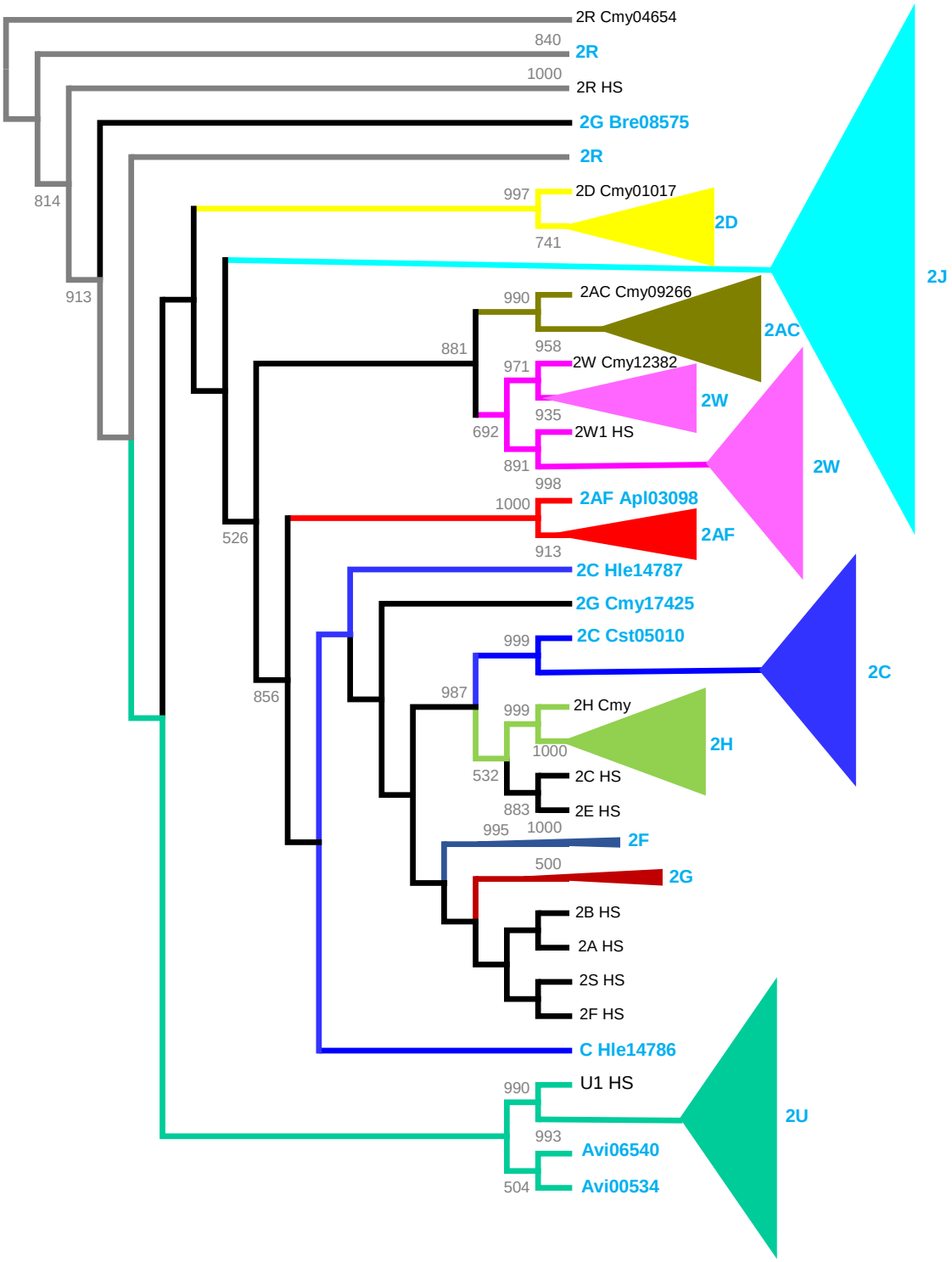



**Figure 18 – Scheme of the Maximum Likelihood tree of the Avian CYP2 family from 45 bird species performed with PhyML v. 3.0 (GTR+G+I, NNIs and 1000 bootstraps). Each one of the CYP2 subfamilies is represented by the corresponding code (2 followed by a capital letter), next the colored clades. The branches are not at scale (length unreal). Blue code – subfamilies from the 45 avian genomes; Black code – subfamilies of representative species from databases.**

Avian cGST family

We also studied the cytosolic GSTs (cGST), one of the detoxification Phase II interveners. cGST are active as homo- or heterodimeric structures (Wu and Dong 2012), each monomer being composed by two major domains. The first one encompassing the N-terminal region, also known as α/β domain or G domain, is the more conserved (Wu and Dong 2012), which could be related with the necessity of a less-variant region crucial for GSH interaction and binding (Sheehan, Meade et al. 2001, Flanagan and Smythe 2011). However, the second domain (known as all-α-helical or H domain) that have as function the accommodation of hydrophobic substrates in order to facilitate their reaction with GSH (Wu and Dong 2012), is less conserved, with variable number of α-helices, which could be justified by the necessity of substrate specificity region in each c (Sheehan, Meade et al. 2001).

In human it was reported the presence of seven classes of cGST genes (alpha, zeta, theta, mu, pi, sigma and omega) (Figure 19), in which gene elements of the same family tend to be clustered in the same chromosomal region (Nebert and Vasiliou 2004, da Fonseca, Johnson et al. 2010, Board and Menon 2013).

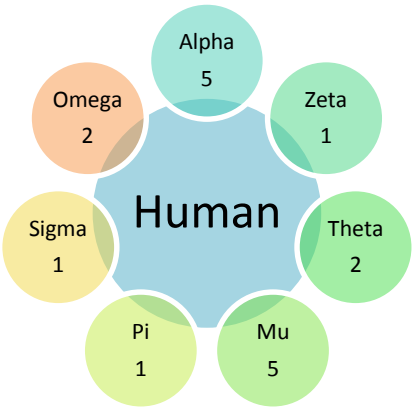



**Figure 19 – Reported cGST classes in human genome, including the detected number of genes inside each class (da Fonseca, Johnson et al. 2010, Board and Menon 2013).**

cGST alpha, mu and theta are involved in cellular reaction connected with metabolism of products resulting from oxidative stress reactions, and usually these class of enzymes have multiples copies in order to provide an efficient rate of fitness against harmful chemicals (da Fonseva, Johnson et al. 2010). More specifically, cGST alpha are related with processing of small hydrophobic molecules (Wu and ODng 2012) but it was also reported a non-detoxification function for the rat GSTA3 like steroid isomerase activity in ovary and testis (Sheehan, Meade et al. 2001, Wu and ODng 2012). In mammals it was reported that cGST mu has a role in AFBO detoxification (Wang, Bammler et al. 2000, Wu and Dong 2012) whereas cGST theta has sulfatase activity

(Wu and Dong 2012) and also affinity for huge amounts of xenobiotic substrates such as halogenated methanes/ethanes and halogenated organic compounds (Landi 2000). Moreover, it was also reported that GST theta can have some affinity for AFBO (Landin 2000).

cGST Pi in humans has a single gene element but two gene copies were reported in mouse (Board and Menon 2013). cGST Pi is involved in protection against genotoxins and neutralization of carcinogenics (Lo, Stephenson et al. 2008), but it has also a high capacity to metabolize polyaromatic hydrocarbons (PAH) metabolites (Knecht, Goodale et al. 2013).

Both cGST sigma and cGST zeta classes have a single gene representative that could be related with their involvement in crucial pathways requiring a tight regulation, and gene duplications might have harmful consequences (da Fonseca, Johnson et al. 2010). For example, cGST sigma is involved in isomerization of prostaglandin H2 to originate prostaglandin D2 (Wu and Dong 2012), which is a lipid with regulatory functions at CNS, respiratory, cardiovascular, genitourinary, endocrine and immune systems (da Fonseca, Johnson et al. 2010). In cGST zeta, it was reported the involvement in the penultimate step of phenylalanine and tyrosine catabolism pathway and one of the reason to require strictly regulation is that changes in degradation of these aromatic amino acids can originate fatal tyrosinemia type I, phenylketonuria or alkaptonuria (da Fonseca, Johnson et al. 2010, Wu and Dong 2012). cGST zeta also catalyzes the biotransformation of several α-holoacids and is involved in important homeostatic reactions (Blackburn, Marrhaei et al. 2006).

cGST omega, that is similar to glutaredoxins due to the use of Cysteine for GSH activation, is duplicated in humans but it is accepted that the ancestral enzyme was involved in ascorbate regeneration (capacity that is always well conserved in cGSTO2) and after duplication, cGSTO1 acquired new capacity to metabolize arsenic (da Fonseca, Johnson et al. 2010). For cGSTO it was also suggested a “housekeeping” function (Sheehan, Meade et al. 2001) and due to their dehydroascorbate reductase activity these enzymes are responsible for maintenance of ascorbic acid levels in brain (Wu and Dong 2012).

Considering their function, the cGST Omega, Zeta and Theta are more related with non-detoxification processes and are ancestral of the cGST Alpha, sigma, Mu and Pi (da Fonseca, Johnson et al. 2010). We used cGST sequences from five bird species available in the Ensembl database to determine which classes of cGSTs are present in avian species and also their phylogenetic relationships.

We found only one gene for the class sigma and zeta, similarly to humans, which support the necessity of a tight regulation of these genes also in birds. In addition to

prostaglandin D2 production, it was reported that chicken cGST sigma has activity toward other molecules such as allyl isothiocyanate and benzyl isothiocyanate molecules, abundant in edible plants like cauliflower, broccoli and cabbage (Thomson, Meyer et al. 1998). In contrast to humans, birds showed only one cGST omega and one cGST theta gene. As in the Ensembl database no cGST mu genes were annotated, we searched the NCBI database and we found one cGST mu gene for chicken and ground tit. However, in turkey we found two distinct genes that are in accordance with the pattern observed in humans. In mammals it was reported that cGST mu has a role in AFBO detoxification (Wang, Bammler et al. 2000, Wu and Dong 2012) but in vitro studies using turkey cGST Mu do not revealed activity in AFBO detoxification (Bunderson, Kim et al. 2013). cGST alpha classes have duplicated genes, with 4 copies in duck, flycatcher and Zebrafinch, five copies in flycatcher and six copies in turkey (Supplementary file 4). cGST alpha duplication scenario in birds could be explained by the affinity of these enzymes for AFBO (Kim, Bunderson et al. 2013). It was suggested previously that cGST Pi is not present in birds (da Fonseca, Johnson et al. 2010) and our extended analysis in 6 avian genomes did not detected this class of cGSTs.

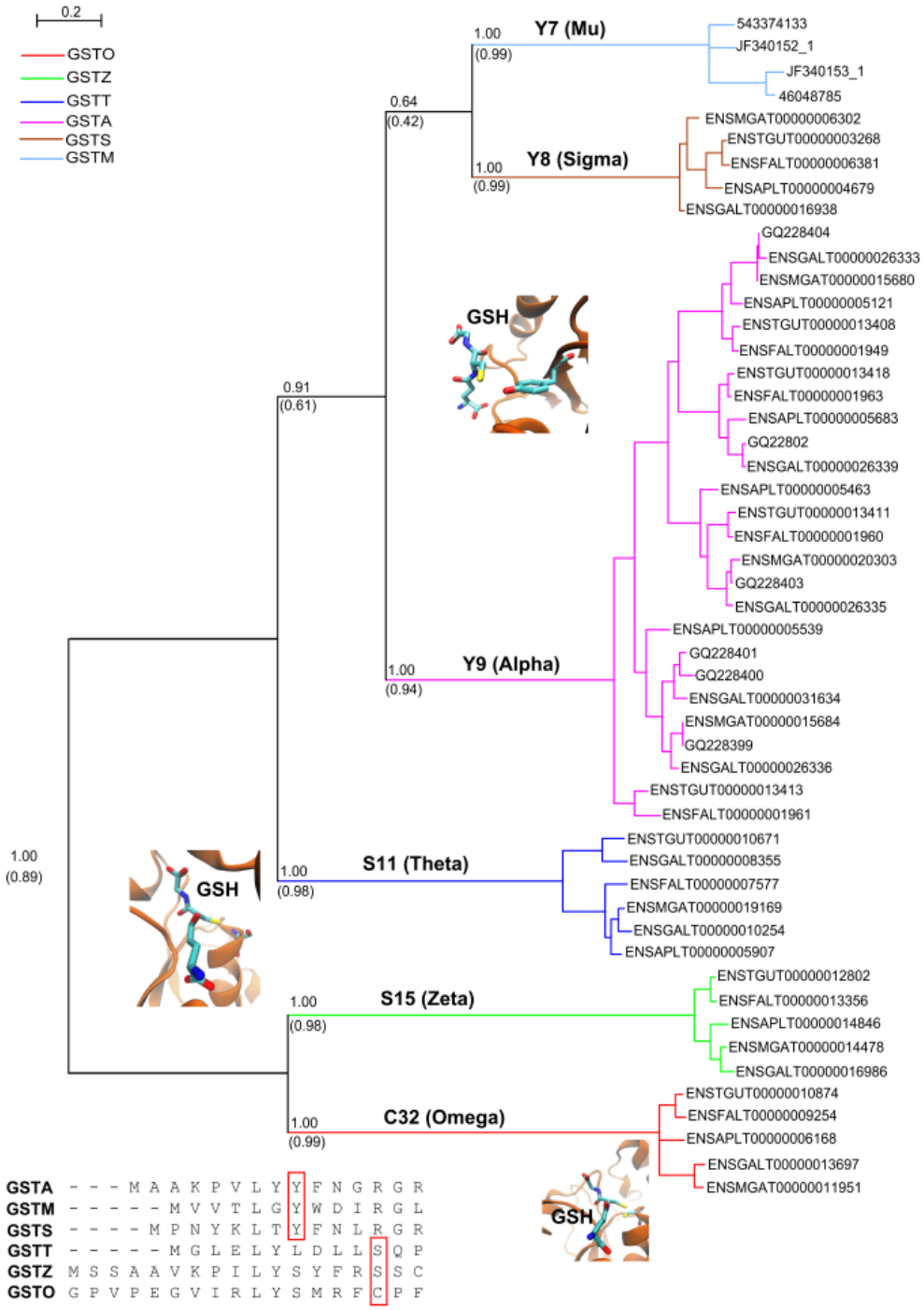



**Figure 19 – Phylogenetic tree of avian cGSTs and alignment of the catalytic residue region.**

The phylogeny of the avian cGSTs (Figure 19) shows that the cGST omega and zeta, referred as basal classes, stay together in a basal clade, whereas cGST theta is also located in a basal position of a clade that include the other cGSTs classes. The phylogeny is also in concordance with the distribution of the catalytic residue. cGST theta and zeta have a serine as catalytic residue, and these two classes should be the more ancestral ones. The serine is responsible for increase the affinity of hydrogen bond with GSH in order to compensate the low affinity of these enzymes for conjugated substrates,

increasing the rate of conversion (Landi 2000). Classes alpha, mu and pi, connected with metabolism of xenobiotic compounds, as also cGST sigma, have a tyrosine as catalytic residue that allows higher affinity and more efficient reactions (Dourado, Fernandes et al. 2008). cGST omega is the only class that presents a cysteine as catalytic residue.

We also analyzed the structure of the avian cGSTs in order to evaluate the conservation degree of the three dimensional cGST structures. The three-dimensional structure of chicken cGST alpha 1 and mu were available in the PDB database (AVF1 and 1GSU, respectively). The three-dimensional structures of other chicken cGST sequences (omega, zeta, theta and sigma) were predicted using the correspondent human cGST structures (1ECM, 1FW1, 2C3N and 2VCZ, respectively). We concluded that the α/β domain is extremely conserved in all cGSTs whereas the majority of variations occur in all-α-helical domain, with helices number varying from 9 in cGST sigma and mu to 12 in cGST theta (Figure 21 and Figure 22).

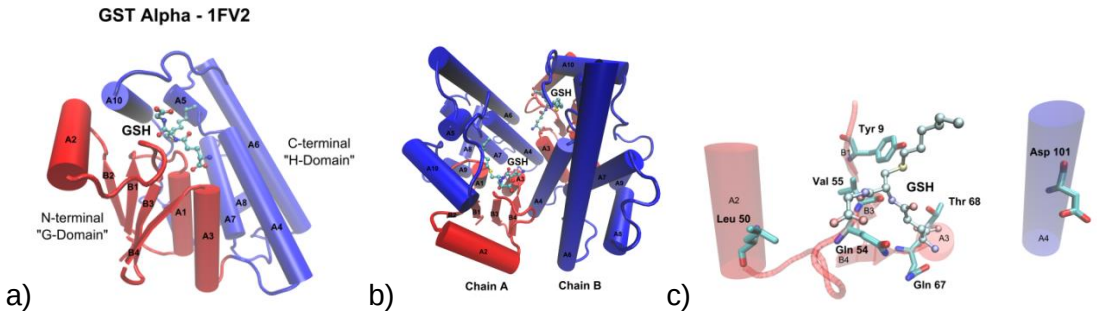



**Figure 21 – Chicken cGST alpha structure. cGSTs possess two domains (a) that interact in complementary domains of other monomers, to form a dimeric structure (b). Some of the more conserved residues involved in GSH stabilization are represented (c).**

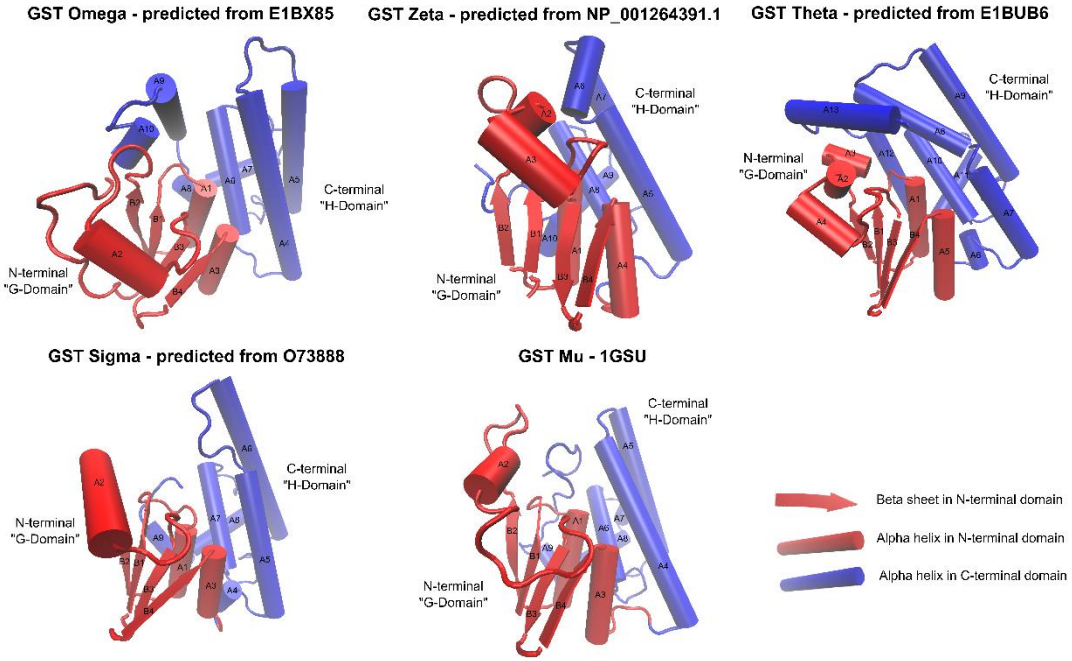



**Figure 22 - Three-dimensional structure of the chicken cGSTs. The G-domain is more conserved than the H-domain.**

cGSTs are involved in the second phase of the detoxification of important environmental pollutants, such as PAH, whereas the first phase of the detoxification is conducted by the CYP450s system (Oakley 2011, Kammann, Brinkmann et al. 2013). However, experimental data did not show correlation between levels of cGST and CYP450 inducible expressions, with cGST level varying much less that in the case of CYP450 enzymes (Forresster, Henderson et al. 1992). This scenario could be explained by the fact that CYP450s are the main system in activation of xenobiotics that can be further metabolized, not only by cGSTs, but also by another huge number of enzymatic systems.

# Conclusions

The study of representative enzymatic systems involved in detoxification in birds had as main goal to understand the evolutionary dynamics of the genes involved in this pathway. Birds are a group of species that present high metabolic rates (Mushi-South and Wilkinson 2010), which should be related with low lifespan. However, birds have almost twice higher lifespan than species of similar size (Montegomery, Hulbert et al. 2011). Moreover, due to the migratory routes and the fact that some birds are in a basal position of the food chain, avian species are often in contact with xenobiotics (Hong, Shim et al. 2014).

Through the analysis of the CYP2 family we detected several duplicated elements in the subfamilies CYP2J and CYP2W. These observations lead us to hypothesize that birds should have developed efficient detoxification systems in order to deal with, both exogenous and endogenous compounds.

Considering the cGST system, despite birds have fewer elements than mammalian species, they should also be extremely functional. One example is the ability of cGST alpha class of wild turkey to metabolize AFBO, conferring an unusual resistance to this mycotoxin (Kim, Bunderson et al. 2013). It was reported that in insects cGSTs have an important role in insecticide resistance (Wu, Dou et al. 2009) and also DDT detoxification (Perera, Hemingway et al. 2008, Che-Mendoza, Penilla et al. 2009). DDT is one of the most common sources of contamination in birds, mainly in piscivorous species (Hong, Shim et al. 2014), that causes mortality and teratogen effects in embryos, reduce fertility, lethal stress and makes eggshell thinning in adult birds, leading to population decreases (Fry 1995). No experimental studies are available about the involvement of avian cGST in DDT detoxification but due to the detected association in insects, it would be interesting in future to analyze if avian cGST are contributing to the DDT elimination.

# General Conclusion

In this work we focus our attention in two examples of biological systems crucial in adaptive processes of species to their environment – sensorial perception system and detoxification system.

In order to ensure the species survival, even in harsh and/or new conditions, the genes are subjected to events of adaptive evolution. In sensorial perception system, we detected that in some species (like rodents, monotremes and marsupials, frogs and snakes) there is a huge number of receptors, which should be related with their importance in detection of a high diversity of pheromones. However, not all species showed that trend, being vomeronasal receptors absent or not yet detected in many vertebrate species. This situation is counter balanced with the use of other receptors for pheromonal identification, such as ORA and OlfC system in fishes, or in other cases the pheromonal communication appear to be replaced by other systems, such as vision, that appear to be more efficient according to the habitat of several species.

In the study of the detoxification system we focus on two key examples in birds, since these group of species present long lifespan even though their high metabolic rates. This contradictory duality could be the result of high efficiently detoxification systems and in our study we detected that birds have a huge number of CYP2 elements from several subfamilies, which increase the diversity of toxic molecules able to be metabolized by CYPs. Even inside the cGST family, birds possess the majority of the reported classes in vertebrates. The synchronized function of all these systems could be the key for a successful detoxification process.

Overall, the well-orchestrated relationship between environment and genes induces dynamic evolutionary modifications, culminating in species adaptation and diversification.

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# Supplementary files

## Supplementary file 1 – ID of characterized cat V1R genes from Ensembl database

ENSFCAG00000030467|ENSFCAT00000029257
ENSFCAG00000030794|ENSFCAT00000031457
ENSFCAG00000031455|ENSFCAT00000029198
ENSFCAG00000029277|ENSFCAT00000031554
ENSFCAG00000025970|ENSFCAT00000029539
ENSFCAG00000022668|ENSFCAT00000024564
ENSFCAG00000028999|ENSFCAT00000022849
ENSFCAG00000007354|ENSFCAT00000007356
ENSFCAG00000031101|ENSFCAT00000024168
ENSFCAG00000031841|ENSFCAT00000030658
ENSFCAG00000026750|ENSFCAT00000028243
ENSFCAG00000026501|ENSFCAT00000028048
ENSFCAG00000025619|ENSFCAT00000025316
ENSFCAG00000022670|ENSFCAT00000032246
ENSFCAG00000029994|ENSFCAT00000025061
ENSFCAG00000029349|ENSFCAT00000024869

# Supplementary file 2 – ID of fish ORA genes from Ensembl and NCBI databases

| | | ORA 1 | ORA 2 | ORA 3 | ORA 4 | ORA 5 | ORA 6 |
|---|---|---|---|---|---|---|---|
| **Coelacanth** | *Latimearia chalumnae* | ENSLACG00000018646<br>ENSLACT00000021365<br>ENSLACG00000018654<br>ENSLACT00000021373<br>ENSLACG00000018660<br>ENSLACT00000021380<br>ENSLACG00000018663<br>ENSLACT00000021383 | ENSLACG00000018669<br>ENSLACT00000021389 | ENSLACG00000019139<br>ENSLACT00000021920<br>ENSLACG00000019138<br>ENSLACT00000021919 | | | |
| **Platyfish** | *Xiphophorus maculatus* | ENSXMAG00000017767<br>ENSXMAT00000017828 | ENSXMAG00000019948<br>ENSXMAT00000020023 | ENSXMAG00000016583<br>ENSXMAT00000016634 | ENSXMAG00000016585<br>ENSXMAT00000016635 | ENSXMAG00000020021<br>ENSXMAT00000020096 | ENSXMAG00000019480<br>ENSXMAT00000019555 |
| **Cichilid** | *Haplochromis chilotes* | 386267893 | 386267894 | 386267896 | 86267897 | 386267898 | 386267900 |
| **Medaka** | *Oryzias latipes* | ENSORLG00000007697<br>ENSORLT00000009642 | ENSORLG00000007694<br>ENSORLT00000009638 | | ENSORLG00000018281<br>ENSORLT00000022895 | ENSORLG00000007638<br>ENSORLT00000009573 | |
| **Tilapia** | *Oreochromis niloticus* | ENSONIG00000020981<br>ENSONIT00000026307 | ENSONIG00000014806<br>ENSONIT00000018658 | ENSONIG00000018921<br>ENSONIT00000023849 | ENSONIG00000018920<br>ENSONIT00000023847 | ENSONIG00000020400<br>ENSONIT00000025726 | ENSONIG00000020917<br>ENSONIT00000026243 |
| **Fugu** | *Takifugu rubripes* | | ENSTRUG00000001765<br>ENSTRUT00000004066 | | | | |
| **Tetraodon** | *Tetraodon nigroviridis* | | ENSTNIG00000010241<br>ENSTNIT00000013334 | | | | |
| **Cod** | *Gadus morhua* | | | ENSGMOG00000008110<br>ENSGMOT00000008924 | ENSGMOG00000008113<br>ENSGMOT00000008925 | ENSGMOG00000020499<br>ENSGMOT00000022504 | ENSGMOG00000020268<br>ENSGMOT00000022273 |
| **Zebrafish** | *Danio rerio* | 194578828 | 148357121 | ENSDARG00000059315<br>ENSDART00000082406<br>ENSDARG00000091638<br>ENSDART00000124720 | ENSDARG00000078223<br>ENSDART00000111873 | ENSDARG00000078257<br>ENSDART00000114666 | ENSDARG00000076078<br>ENSDART00000110558 |
| **Cavefish** | *Astyanax mexicanus* | ENSAMXG00000024873<br>ENSAMXT00000025599 | ENSAMXG00000008360<br>ENSAMXT00000008590 | ENSAMXG00000004763<br>ENSAMXT00000004874 | ENSAMXG00000008688<br>ENSAMXT00000008926 | ENSAMXG00000025741<br>ENSAMXT00000026467 | ENSAMXG00000006134<br>ENSAMXT00000006293 |
| **Spotted gar** | *Lepisosteus oculatus* | | ENSLOCG00000017935<br>ENSLOCT00000022077 | ENSLOCG00000007740<br>ENSLOCT00000009409 | ENSLOCG00000007709<br>ENSLOCT00000009365 | ENSLOCG00000017551<br>ENSLOCT00000021693 | ENSLOCG00000017890<br>ENSLOCT00000022032 |
| **Lamprey** | *Petromyzon marinus* | | | ENSPMAG00000010425<br>ENSPMAT00000011452 | | | |

Underlined sequences correspond to ORA1-like genes

# Supplementary file 3

## ID of CaSR genes from Ensembl database

ENSDARG00000013649_ENSDART00000010934
ENSGACG00000020687_ENSGACT00000027407
ENSGMOG00000002672_ENSGMOT00000002894
ENSLACG00000008044_ENSLACT00000009182
ENSLOCG00000002893_ENSLOCT00000003416
ENSONIG00000005168_ENSONIT00000006501
ENSTNIG00000010032_ENSTNIT00000013119
ENSTRUG00000016870_ENSTRUT00000043375

## *Danio rerio* OlfC genes reported by Hashiguchi and Nashida 2006 and used in analyzes

| | | | | | | |
|---|---|---|---|---|---|---|
| D02 | H02 | H07 | H11 | H15 | H23 | J02 |
| E01 | H03 | H08 | H12 | H16 | H24 | K01 |
| G01 | H04 | H09 | H13 | H17 | I01 | |
| H01 | H05 | H10 | H14 | H21 | J01 | |

## ID of OlfC genes from Ensembl database

ENSAMXG00000016664|ENSAMXT00000017162 – V2RL1
ENSAMXG00000019881|ENSAMXT00000020463 – OlfCW1.1
ENSAMXG00000019921|ENSAMXT00000020510
ENSAMXG00000019933|ENSAMXT00000020520
ENSAMXG00000019941|ENSAMXT00000020525 – OlfCH1
ENSAMXG00000019951|ENSAMXT00000020536 – OlfCJ1
ENSAMXG00000019989|ENSAMXT00000020569
ENSAMXG00000019993|ENSAMXT00000020574
ENSAMXG00000020009|ENSAMXT00000020596
ENSAMXG00000020019|ENSAMXT00000020603
ENSAMXG00000020097|ENSAMXT00000020689
ENSAMXG00000020101|ENSAMXT00000020691
ENSAMXG00000020182|ENSAMXT00000020786
ENSDARG00000004975|ENSDART00000145023 – OlfCG9
ENSDARG00000008095|ENSDART00000099094 – OlfCD1
ENSDARG00000011034|ENSDART00000057845
ENSDARG00000011604|ENSDART00000067574 – OlfCG1
ENSDARG00000022229|ENSDART00000059643
ENSDARG00000025351|ENSDART00000137393
ENSDARG00000027697|ENSDART00000139869 – OlfCW1
ENSDARG00000027720|ENSDART00000067567 – OlfCG5
ENSDARG00000029635|ENSDART00000133276
ENSDARG00000033266|ENSDART00000140909 – OlfCS2
ENSDARG00000035214|ENSDART00000140043
ENSDARG00000038532|ENSDART00000088854
ENSDARG00000040594|ENSDART00000048182
ENSDARG00000040632|ENSDART00000019233
ENSDARG00000043686|ENSDART00000067572 – OlfCG2
ENSDARG00000053075|ENSDART00000050178 – OlfCH11
ENSDARG00000055920|ENSDART00000145000
ENSDARG00000055949|ENSDART00000099219 – OlfCD2
ENSDARG00000055983|ENSDART00000007035 – OlfCH12
ENSDARG00000055994|ENSDART00000140885 – OlfCH1
ENSDARG00000056000|ENSDART00000059412 – OlfCH7
ENSDARG00000056006|ENSDART00000135254
ENSDARG00000056009|ENSDART00000022155 – OlfCH9
ENSDARG00000056014|ENSDART00000099188
ENSDARG00000056097|ENSDART00000143140 – OlfCS1
ENSDARG00000056180|ENSDART00000132758
ENSDARG00000061883|ENSDART00000153946 – OlfCH10
ENSDARG00000061903|ENSDART00000136603
ENSDARG00000067828|ENSDART00000003907
ENSDARG00000068520|ENSDART00000099139 – OlfCH14
ENSDARG00000068549|ENSDART00000139117
ENSDARG00000068566|ENSDART00000131788 – OlfCQ19
ENSDARG00000068576|ENSDART00000059500 – OlfCD3
ENSDARG00000073780|ENSDART00000056223
ENSDARG00000075988|ENSDART00000104792 – OlfCH1
ENSDARG00000079535|ENSDART00000003955
ENSDARG00000079954|ENSDART00000004952 – OlfCJ1
ENSDARG00000088029|ENSDART00000064153
ENSDARG00000088192|ENSDART00000099142 – OlfCH13
ENSDARG00000091790|ENSDART00000099184
ENSDARG00000091990|ENSDART00000131587
ENSDARG00000092523|ENSDART00000136070
ENSDARG00000096362|ENSDART00000151623
ENSGACG00000007060|ENSGACT00000009373 – V2RL1
ENSGACG00000010313|ENSGACT00000013649 – OlfCJ1
ENSGACG00000010316|ENSGACT00000013656 – OlfCH1
ENSGACG00000010323|ENSGACT00000013663
ENSGACG00000010329|ENSGACT00000013676
ENSGACG00000010337|ENSGACT00000013682

ENSGACG00000010342|ENSGACT00000013685
ENSGACG00000010344|ENSGACT00000013692
ENSGACG00000010349|ENSGACT00000013713
ENSGACG00000010364|ENSGACT00000013722
ENSGMOG00000009045|ENSGMOT00000009926 – V2RL1
ENSGMOG00000014833|ENSGMOT00000016300
ENSGMOG00000015132|ENSGMOT00000016615
ENSGMOG00000015158|ENSGMOT00000016663
ENSGMOG00000015461|ENSGMOT00000016998
ENSLACG00000003042ENSLACT00000003438
ENSLACG00000003260ENSLACT00000003694
ENSLACG00000003887ENSLACT00000004411
ENSLACG00000004180ENSLACT00000004739
ENSLACG00000004927ENSLACT00000005591 – OlfCW1
ENSLOCG00000002396|ENSLOCT00000002814
ENSLOCG00000002450|ENSLOCT00000002889
ENSLOCG00000002475|ENSLOCT00000002923
ENSLOCG00000002500|ENSLOCT00000002946
ENSLOCG00000002552|ENSLOCT00000003025
ENSLOCG00000002596|ENSLOCT00000003056 – OlfCJ1.1
ENSLOCG00000002611|ENSLOCT00000003074 – OlfCJ1.2
ENSLOCG00000002648|ENSLOCT00000003114 – OlfCJ1.3
ENSLOCG00000002660|ENSLOCT00000003134 – OlfCJ1.4
ENSLOCG00000002751|ENSLOCT00000003244
ENSLOCG00000002766|ENSLOCT00000003262
ENSLOCG00000002816|ENSLOCT00000003319 – OlfCH1
ENSLOCG00000002830|ENSLOCT00000003345
ENSLOCG00000002906|ENSLOCT00000003439
ENSLOCG00000002932|ENSLOCT00000003461
ENSLOCG00000002973|ENSLOCT00000003508
ENSLOCG00000003038|ENSLOCT00000003578
ENSLOCG00000003047|ENSLOCT00000003625
ENSLOCG00000003084|ENSLOCT00000003655
ENSLOCG00000003112|ENSLOCT00000003677
ENSLOCG00000003230|ENSLOCT00000003823 – V2RL1
ENSONIG00000003508|ENSONIT00000004419
ENSONIG00000005521|ENSONIT00000006944 – V2RL1
ENSONIG00000010895|ENSONIT00000013720 – OlfCH1
ENSONIG00000010897|ENSONIT00000013722
ENSONIG00000010898|ENSONIT00000013724
ENSONIG00000010901|ENSONIT00000013727
ENSONIG00000010909|ENSONIT00000013737
ENSONIG00000010917|ENSONIT00000013745
ENSONIG00000010922|ENSONIT00000013751
ENSONIG00000010923|ENSONIT00000013752
ENSONIG00000011187|ENSONIT00000014092
ENSONIG00000011190|ENSONIT00000014095
ENSONIG00000011192|ENSONIT00000014097
ENSONIG00000011195|ENSONIT00000014100
ENSONIG00000011205|ENSONIT00000014112
ENSONIG00000011212|ENSONIT00000014120
ENSONIG00000011213|ENSONIT00000014121
ENSONIG00000011218|ENSONIT00000014127
ENSONIG00000011221|ENSONIT00000014130
ENSONIG00000011222|ENSONIT00000014131
ENSONIG00000011224|ENSONIT00000014133
ENSORLG00000005562|ENSORLT00000006999
ENSORLG00000005577|ENSORLT00000007015
ENSORLG00000005587|ENSORLT00000007028
ENSORLG00000005596|ENSORLT00000007037
ENSORLG00000005639|ENSORLT00000007086
ENSORLG00000005648|ENSORLT00000007102
ENSORLG00000005658|ENSORLT00000007115
ENSORLG00000005678|ENSORLT00000007140
ENSORLG00000005695|ENSORLT00000007159 – OlfC H1.1
ENSORLG00000005700|ENSORLT00000007165 – OlfCH1.2
ENSORLG00000005715|ENSORLT00000007184 – OlfCG9
ENSORLG00000005734|ENSORLT00000007208
ENSORLG00000012554|ENSORLT00000015716 – V2RL1
ENSTNIG00000001733|ENSTNIT00000001948
ENSTNIG00000003340|ENSTNIT00000004435 – V2RL1
ENSTNIG00000003540|ENSTNIT00000006276
ENSTNIG00000003568|ENSTNIT00000006306
ENSTNIG00000007683|ENSTNIT00000006277
ENSTNIG00000007687|ENSTNIT00000010684 – OlfCH1
ENSTNIG00000007689|ENSTNIT00000002938
ENSTRUG00000000503|ENSTRUT00000001200
ENSTRUG00000001137|ENSTRUT00000002662
ENSTRUG00000002224|ENSTRUT00000005133
ENSTRUG00000002837|ENSTRUT00000006657
ENSTRUG00000004252|ENSTRUT00000010127
ENSTRUG00000004327|ENSTRUT00000010325
ENSTRUG00000006589|ENSTRUT00000016257 – V2RL1
ENSTRUG00000007182|ENSTRUT00000017736
ENSTRUG00000008774|ENSTRUT00000022131
ENSTRUG00000014084|ENSTRUT00000036172

# Supplementary file 4 – ID of avian cGST genes from Ensembl and NCBI databases

| Species | ID | GST class |
|---|---|---|
| Duck *Anas platyrhynchos* | ENSAPLG00000005723\|ENSAPLT00000005907 | T1 |
| | ENSAPLG00000004536\|ENSAPLT00000004679 | S |
| | ENSAPLG00000004953\|ENSAPLT00000005121 | A4 |
| | ENSAPLG00000005277\|ENSAPLT00000005463 | A3 |
| | ENSAPLG00000005354\|ENSAPLT00000005539 | A1 |
| | ENSAPLG00000005503\|ENSAPLT00000005683 | A2 |
| | ENSAPLG00000005953\|ENSAPLT00000006168 | O1 |
| | ENSAPLG00000014216\|ENSAPLT00000014846 | Z |
| Flycatcher *Ficedula albicollis* | ENSFALG00000001877\|ENSFALT00000001963 | A2 |
| | ENSFALG00000001862\|ENSFALT00000001949 | A4 |
| | ENSFALG00000001868\|ENSFALT00000001960 | A3 |
| | ENSFALG00000001874\|ENSFALT00000001961 | A1 |
| | ENSFALG00000006093\|ENSFALT00000006381 | S |
| | ENSFALG00000007230\|ENSFALT00000007577 | T1 |
| | ENSFALG00000008832\|ENSFALT00000009254 | O1 |
| Chicken *Gallus gallus* | ENSGALG00000016322\|ENSGALT00000026333 | A4 |
| | ENSGALG00000005204\|ENSGALT00000008355 | T1L |
| | ENSGALG00000006344\|ENSGALT00000010254 | T1 |
| | ENSGALG00000008409\|ENSGALT00000013697 | O1 |
| | ENSGALG00000010402\|ENSGALT00000016938 | S |
| | ENSGALG00000010432\|ENSGALT00000016986 | Z |
| | ENSGALG00000016324\|ENSGALT00000026335 | A3 |
| | ENSGALG00000016325\|ENSGALT00000026336 | A1 |
| | ENSGALG00000016328\|ENSGALT00000026339 | A2 |
| | ENSGALG00000028551\|ENSGALT00000031634 | A1 |
| | gi\|46048785 | M2 |
| Turkey *Meleagris gallopavo* | JF340153.1\|:1-663 | M |
| | JF340152.1\|:1-660 | M |
| | GQ228399.1_GSTA1_1 | A1_1 |
| | GQ228400_GSTA1_2 | A1_2 |
| | GQ228401_GSTA1_3 | A1_3 |
| | GQ228402_GSTA2 | A2 |
| | GQ228403_GSTA3 | A3 |
| | GQ228404_GSTA4 | A4 |
| | ENSMGAG00000013933\|ENSMGAT00000015680 | A4 |
| | ENSMGAG00000005610\|ENSMGAT00000006302 | S |
| | ENSMGAG00000007569\|ENSMGAT00000019169 | T1 |
| | ENSMGAG00000010660\|ENSMGAT00000011951 | O1 |
| | ENSMGAG00000012865\|ENSMGAT00000014478 | Z |
| | ENSMGAG00000013935\|ENSMGAT00000020303 | A3 |
| | ENSMGAG00000013939\|ENSMGAT00000015684 | A1 |
| Zebra finch *Taeniopygia guttata* | ENSTGUG00000010242\|ENSTGUT00000010671 | T1L1 |
| | ENSTGUG00000003144\|ENSTGUT00000003268 | S |
| | ENSTGUG00000010435\|ENSTGUT00000010874 | O |
| | ENSTGUG00000012291\|ENSTGUT00000012802 | Z |
| | ENSTGUG00000012876\|ENSTGUT00000013408 | A4 |
| | ENSTGUG00000012878\|ENSTGUT00000013411 | A3 |
| | ENSTGUG00000012881\|ENSTGUT00000013413 | A1 |
| | ENSTGUG00000012882\|ENSTGUT00000013418 | A2 |
| Ground Tit *Pseudopodoces humilis* | gi\|543374133\|ref\|XM_005530225.1\| | M |